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Autophagy beyond convention: plant-specific mechanisms for cellular recycling

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Abstract

Autophagy is an evolutionarily conserved catabolic pathway in eukaryotes, mediated by AuTophaGy related proteins (ATGs). It serves as a housekeeping mechanism by degrading diverse cellular components ranging from protein aggregates to entire organelles. Autophagy can either execute bulk degradation or exhibit high selectivity in targeting its cargo in response to the prevailing environmental conditions. In this thesis, we began by investigating adaptations of autophagy in the autotrophic and heterotrophic organs of plants. Our study revealed organ-specific dynamics of autophagic response in *Arabidopsis thaliana* under different stress conditions, with roots consistently exhibiting an earlier and more robust activity than shoots. We further identified the spatiotemporal autophagic selectivity towards different organelles under bulk autophagy-inducing conditions. In an effort to uncover distinctions in the autophagy machinery responsible for these plant-specific autophagic responses, we discovered that the ATG4-mediated delipidation of ATG8, previously considered a fundamental step in all eukaryotes, is dispensable in *Arabidopsis thaliana*. Further investigation into the plant ATG proteins led to the discovery of their potential roles beyond autophagy, as exemplified by the interactome of ATG5 which includes, among others, proteins involved in the endomembrane trafficking system and components of the ubiquitin-proteasome system. We developed two specialized tools to enable the above studies: SPIRO, an automated time-lapse imaging system designed for conducting phenotypical assays, and RoPod, dedicated microscopy chambers that aid in low-stress imaging of Arabidopsis roots.

Keywords: Arabidopsis, roots, shoots, autophagy, stress, selectivity, ATG4, ATG8, ATG5, tools

Autofagi bortom konventionen: växtspecifika mekanismer för cellulär återvinning

Sammanfattning

Autofagi är en evolutionärt bevarad katabolisk reaktionsväg hos eukaryoter, medierad av AuTophaGy-relaterade proteiner (ATGs). Autofagi fungerar som en underhållsmekanism genom att bryta ner olika cellulära komponenter, från proteinaggregat till hela organeller. Autofagi kan antingen utföra bulknedbrytning eller visa hög selektivitet till cellmaterial, beroende på de rådande miljöförhållandena. I denna avhandling började vi med att undersöka anpassningar av autofagi i växternas autotrofa och heterotrofa organ. Vår studie kunde avslöja den organspecifika dynamiken i autofagisk respons i *Arabidopsis thaliana* under olika stressförhållanden, där rötter konsekvent visar en tidigare och kraftigare aktivitet än skott. Vi identifierade även den rumsliga och tidsmässiga autofagiska selektiviteten gentemot olika organeller i de två organen under bulk autofagi-inducerande förhållanden. I ett försök att avslöja skillnader i autofagimaskineriet som är ansvarigt för dessa växtspecifika autofagiska responser, upptäckte vi att ATG4-medierad avlipidering av ATG8, tidigare betraktad som ett grundläggande steg hos alla eukaryoter, är onödig i Arabidopsis thaliana. Vidare forskning om växtens ATG-proteiner ledde till upptäckten av deras potentiella roller bortom autofagi, exemplifierat med interaktomet för ATG5, som bland annat inkluderar proteiner som är involverade i det endomembrana trafiksystemet och komponenter av ubiquitin-proteasomsystemet. Vi utvecklade två specialverktyg för att möjliggöra ovanstående studier: SPIRO, ett automatiserat tidsförloppsbildningssystem utformat för att utföra fenotypiska tester, och RoPod, en mikroskopikammare designad för att minimiera stresspåverkan av Arabidopsis-rötter.

Nyckelord: Arabidopsis, rötter, skott, autofagi, stress, selektivitet, ATG4, ATG8, ATG5, verktyg

Dedication

To my beloved family...

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Sanjana Holla, Jonas A Ohlsson, Francis Impens, Teresa Maia, Evy Timmerman, Yasin Dagdas, Christian Löfke, Kerstin Dalman, Adrian N Dauphinee, Karin Schumacher, Peter V Bozhkov, and Elena A Minina. Plant autophagy exhibits organ-specific dynamics and selectivity. *(manuscript)*

II. Sanjana Holla, Yong Zou, Igor Sabljic, Jonas A Ohlsson, Jia Xuan Leong, Florentine Ballhaus, Melanie Krebs, Karin Schumacher, Suayib Üstün, Yasin Dagdas, Peter V Bozhkov, and Elena A Minina. Diversified Roles of ATG8-Delipidation in Autophagy Across Eukaryotic Lineages. *(manuscript)*

III. Pernilla H. Elander*, Sanjana Holla*, Igor Sabljić, Emilio Gutierrez-Beltran, Patrick Willems, Peter V. Bozhkov, and Elena A. Minina. 2023. Interactome of *Arabidopsis* ATG5 Suggests Functions beyond Autophagy. *International Journal of Molecular Sciences* 24, no. 15: 12300.

*These authors contributed equally.

IV. Jonas A Ohlsson, Jia Xuan Leong, Pernilla H Elander, Florentine Ballhaus, Sanjana Holla, Adrian N Dauphinee, Johan Johansson, Mark Lommel, Gero Hofmann, Staffan Betnér, Mats Sandgren, Karin Schumacher, Peter V Bozhkov, and Elena A Minina. SPIRO – the automated Petri plate imaging platform designed by biologists, for biologists. *BioRxiv, submitted*

V. Marjorie Guichard, Sanjana Holla, Daša Wernerová, Guido Grossmann, and Elena A. Minina. RoPod, a customizable toolkit for non-invasive root imaging, reveals cell type-specific dynamics of plant autophagy. *BioRxiv, in revision*

The contribution of Sanjana Holla to the papers included in this thesis was as follows:

I. Designed and performed experiments, analysed data except mass-spectrometry, and generated figures. Contributed in writing the manuscript.

II. Contributed in performing experiments and analysing data.

III. Contributed in performing experiments, analysing data, generating figures, and writing the manuscript.

IV. Contributed in performing proof of concept experiments.

V. Contributed in performing proof of concept experiments.

List of figures

Abbreviations

–C	Carbon starvation
–N	Nitrogen starvation
Ab	Autophagic body
AIM	ATG8 Interacting Motif
ATG	Autophagy-related protein/gene
CCV	Clathrin Coated Vesicles
COPII	Coat Protein Complex II
ER	Endoplasmic Reticulum
ERES	Endoplasmic Reticulum Exit Site
ERGIC	ER-Golgi Intermediate Compartment
ESCRT	Endosomal Sorting Complex Required for Transport
FIP200	Focal adhesion kinase family Interacting Protein of 200 kD
GA	Golgi Apparatus
GFP	Green Fluorescent Protein
LECA	Last Eukaryotic Common Ancestor
MCS	Membrane Contact Sites
MVB	Multi-Vesicular Body
PAS	Phagophore Assembly Site

PE	Phosphatidylethanolamine
PI3K	Phosphoinositide 3-Kinase
PI3P	Phosphatidylinositol-3-phosphate
PM	Plasma Membrane
SAR	Selective Autophagy Receptor
SPIRO	Smart Plate Imaging Robot
TEM	Transmission Electron Microscope
TGN	Trans-Golgi Network
TOR	Target of Rapamycin
Ub	Ubiquitin
Ubl	Ubiquitin-like
ULK1	Unc51-like Kinase 1
UPS	Ubiquitin Proteasome System
VPS	Vacuolar Protein Sorting
WIPI	WD-repeat protein Interacting with Phosphoinositide

1. Introduction

Autophagy is a fundamental cellular degradation and recycling strategy adopted by eukaryotes. This chapter aims to guide the reader through the evolutionary trajectory of autophagy, starting with its origin and advancing to the mechanisms governing this process. Emphasis is placed on discerning the similarities and differences in this process between unicellular and multicellular organisms. Particular focus is on highlighting the plant-specific features of autophagy, which will serve as the foundation for comprehending the findings presented in this thesis. Further, the chapter delves into the crosstalk between autophagy and other essential cellular processes, unveiling how autophagy's dynamic interplay with these processes shapes its adaptation. When providing examples from yeast and plants, the focus will be on *Saccharomyces cerevisiae* and *Arabidopsis thaliana,* respectively, unless stated otherwise.

"*Nothing in biology makes sense except in the light of evolution*" - Theodosius Dobzhansky

1.1 From prokaryotes to eukaryotes: the emergence of complexity

To comprehend the mechanisms governing cellular processes, it can be beneficial to trace the evolutionary journey that has sculpted the cellular landscape. Life on Earth began approximately 3.7 billion years ago with the emergence of prokaryotes (Nutman et al., 2016). Prokaryotes, exemplified by *Bacteria* and *Archaea* (Schleifer, 2009), are unicellular organisms that lack a true nucleus and membrane-bound organelles.

The transition from prokaryotes to eukaryotes, known as eukaryogenesis (López-García & Moreira, 2019), stands as one of the most fascinating events in the history of life on Earth. While the exact mechanism of this process is still debated, various theories have provided valuable insights (López-García & Moreira, 2015). The widely accepted endosymbiotic theory suggests that eukaryotic cells evolved from symbiotic relationships, with mitochondria and plastids originating from alphaproteobacteria and cyanobacteria, respectively. The serial endosymbiosis hypothesis extends this idea to multiple rounds of organelle acquisition, while other theories propose mechanisms like phagocytosis and plasma membrane (PM) infoldings as contributors to the emergence of eukaryotic complexity (Bell, 2022). Without indulging further into the debate, it is fair to say that eukaryotes gained significant advantages over their prokaryotic counterparts. This includes the acquisition of membrane-bound organelles that compartmentalize cellular functions, including a true nucleus housing their genetic material, the presence of mitochondria, and, in photosynthetic eukaryotes, chloroplasts for energy production; as well as a complex cytoskeleton for structural support. Notable advantages came from their adoption of an extensive endomembrane system regulating protein and lipid synthesis and transport. This feat can be traced back to the Last Eukaryotic Common Ancestor (LECA), a hypothetical lineage representing the shared ancestry from which all modern eukaryotic organisms have diverged.

1.2 The endomembrane system

Central to the evolution of eukaryotes is the development of the endomembrane system. This system consists of a group of organelles that facilitate the production, modification, packaging, and transport of lipids and proteins in the cell. Organelles considered to be a part of the endomembrane system include the Endoplasmic Reticulum (ER), Golgi Apparatus (GA). Trans-Golgi Network (TGN)/ Early Endosomes (EE), PM, Multi Vesicular Bodies (MVBs), and the lytic compartments: lysosomes in mammals, and vacuoles in yeast and plants. Additionally, some studies also claim that the peroxisomes are an integral part of this system (Beach et al., 2012).

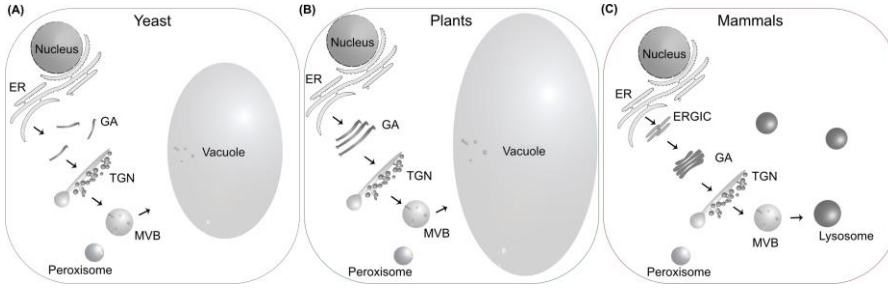


Figure 1. Structural differences in the organelles of the endomembrane system in yeast, plants, and mammals. (A) In yeast, the Golgi Apparatus (GA) consists of dispersed cisterna, (B) whereas in plants, they are a stack of motile flattened cisternae. (C) In mammals, the GA is represented by sessile stacks of ribbon-like cisternae. Additionally, in mammals, proteins and lipids synthesized in the Endoplasmic reticulum (ER), are trafficked to the GA via the ER-Golgi Intermediate Compartments (ERGIC) and end up in the small lytic lysosomes. TGN- Trans-Golgi network, MVB- Multi-vesicular bodies.

While the overall organization of the endomembrane system is conserved among eukaryotes, specific adaptations are observed in different organisms. For example, GA in yeast (unicellular organisms) consists of dispersed individual cisterna (Papanikou & Glick, 2009) (Figure 1A), while in plants and mammals, they exist either as motile stacks of flattened cisternae (Dupree & Sherrier, 1998; Robinson, 2020) (Figure 1B) or static ribbon-like cisternae (Benvenuto et al., 2023) (Figure 1C), respectively. Additionally, in mammals, protein-laden vesicles traverse from the ER to GA via the ER-exit sites (ERES) and ER-Golgi Intermediate compartment (ERGIC) (Figure 1C), while in yeast and plants, proteins are directly transferred to the GA from the ERES (Sparkes et al., 2009; Takagi et al., 2020). In terms of the lytic compartments, yeast and plants possess a single large immobile vacuole (Figure 1A, 1B), whereas mammals have multiple small motile lysosomes (Wada, 2013) (Figure 1C). The structural differences in organelles between these organisms reflect the specific evolutionary adaptations, functional requirements, and environmental demands.

1.3 Cellular house-keeping

Owing to the acquisition of complex cellular organization in eukaryotic cells, it is conceivable that they would require extensive housekeeping mechanisms. One such mechanism is the Ubiquitin Proteasome System (UPS), potentially carried over from their prokaryotic ancestors, as

evidenced by the discovery of UPS components in *Bacteria* and *Archaea* (Burns et al., 2009; Nunoura et al., 2011). The UPS is a major protein degradation process involved in eliminating short-lived, damaged, and misfolded proteins. Proteins to be degraded are marked with ubiquitin (Ub) by the concerted action of ubiquitin-activating enzymes (E1), ubiquitin-transferring enzymes (E2), and ubiquitin ligases (E3). The 26S proteasome is the protease machinery responsible for clearing the proteins tagged for degradation (Nandi et al., 2006). In eukaryotes, while misfolded and short-lived proteins are degraded by the UPS, a need may have arisen for a mechanism to degrade macromolecules, including degenerated or damaged organelles within the cells: thereby introducing us to the process of 'Autophagy'.

1.4 Autophagy

Coined by the Belgian biochemist, Christian de Duve in 1963, autophagy originates from the Greek words "auto" (self) and "phagy" (eating) (Klionsky, 2008). Unlike its UPS counterpart, autophagy is an evolutionarily conserved process found exclusively in eukaryotes, as marked by its presence in LECA (S. Zhang et al., 2021). There are primarily three types of autophagy- chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. CMA has only been identified in mammals, and it involves the direct (vesicle-independent) transport of cytoplasmic content (cargo) into the lysosomes (Tasset & Cuervo, 2016). Microautophagy is characterized by the invagination of lysosome/vacuole membrane and vesicle-independent internalization of cargo into the lumen for degradation (Sieńko et al., 2020; L. Wang et al., 2023). Macroautophagy (hereafter referred to as autophagy) is the most well-studied process of all. It is mediated via the formation of double-membraned structures called autophagosomes, which engulf and deliver cytoplasmic cargo, including damaged organelles and protein aggregates to the lytic compartments. Within these compartments, resident hydrolases break down the delivered cargo, and the resulting degradation products are recycled back into the cytoplasm.

1.4.1 Origin of autophagy proteins

Prokaryotic precursors

Despite the fact that autophagy is unique to eukaryotes, it is unlikely from an evolutionary stance that this catabolic process emerged abruptly in the first eukaryotic organism. To delve into this further, we shift our focus to the proteins responsible for governing autophagy, known as AuTophaGy-related (ATG) genes/proteins. Interestingly enough, studies have revealed the ancestral roots of ATGs in prokaryotes, substantiated by the existence of remote homologs within them. For example, the sulfur carrier proteins, ThiS and MoaD, conserved in most prokaryotes, possess a β-grasp fold, similar to the Ubs found in eukaryotes (Burroughs et al., 2007). In autophagy, essential proteins ATG12 and ATG8 are ubiquitin-like (Ubl) proteins that also comprise the β-grasp fold (Cappadocia & Lima, 2018; S. Zhang et al., 2021). Another example highlighting the functional preservation across prokaryotes and eukaryotes lies in the chorein-N domain. This domain, responsible for lipid transfer in prokaryotes has been evolutionarily conserved in the ATG2 proteins (S. Zhang et al., 2021). These examples, along with the other insights presented in Zhang et al., 2021, indicate that some components of autophagy have a prokaryotic lineage, and may have gradually refined and adapted to 'additionally' serve in the catabolic process. Furthermore, these examples and insights also highlight the tight interlink between autophagy and the UPS, adding an interesting essence to the story.

Multigene families

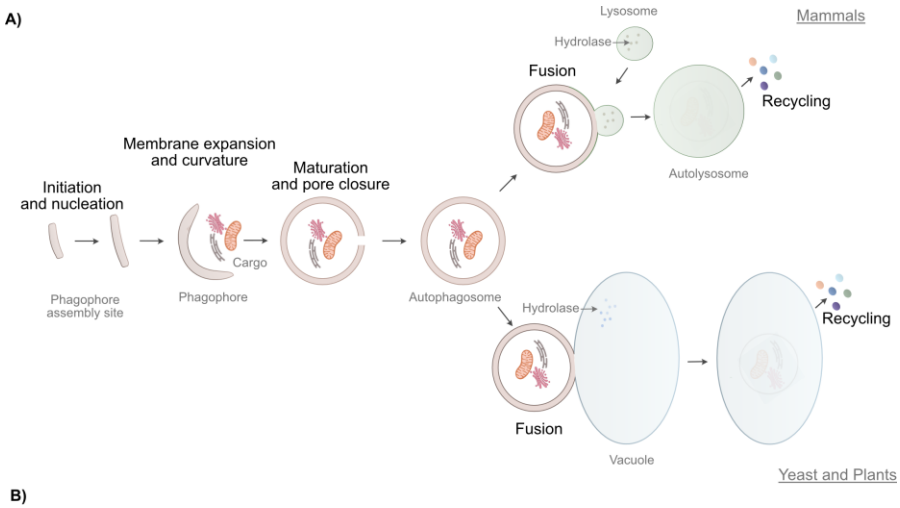
Although autophagy was initially discovered in mammals, it was through yeast genetic screens that ATGs were originally identified (Tsukada & Ohsumi, 1993). Currently, over 40 ATGs have been characterized in yeast, with approximately 20 core proteins involved in autophagosome biogenesis (Nakatogawa, 2020; Wen & Klionsky, 2016). Most of the ATG proteins identified in yeast are conserved, with homologs found in plants (Marshall & Vierstra, 2018) and mammals (Nakatogawa, 2020). It is interesting to note here that yeasts possess single-member ATG gene families, while in multicellular organisms, certain gene families are characterized by multiple isoforms (S. Zhang et al., 2021). The acquisition of multigene families in these organisms could reflect their adaptation to meet the increased metabolic needs and cater to the specific demands for higher-order functions of different cell types, tissues, and organs. Moreover, it suggests that these

isoforms may have specialized functions in autophagy, as elucidated in Paper II, with the ATG8 isoforms.

1.4.2 The autophagy machinery

Autophagosome biogenesis is a complex and highly regulated process. Briefly, during autophagosome biogenesis, a small, flattened membrane structure called the 'isolation membrane' or 'phagophore' emerges in the cytoplasm, which undergoes expansion and curvature, ultimately becoming spherical. After pore closure, the formation of the double-membrane autophagosome is completed. This autophagosome is subsequently transported to the vacuole/lysosome. Upon reaching its destination, the outer autophagosomal membrane fuses with the vacuolar/lysosomal membrane. Within these lytic organelles, the inner autophagosomal membrane and sequestered materials undergo degradation. The degradation products are then transported back to the cytoplasm, where they are recycled for various cellular functions (Y. Hu & Reggiori, 2022; Zhuang et al., 2018) (Figure 2A). The autophagosome biogenesis involves a set of core ATG proteins (Figure 2B). These include (i) the ATG1/Unc-51 Like Autophagy Activating Kinase 1 (ULK1) complex (ii) members of the class III phosphatidylinositol 3-kinase (PI3K) complex, (iii) ATG9 and its cycling system, (iv) ATG12 conjugation system, and (v) ATG8 conjugation system. These core proteins are conserved in most eukaryotes, except *red algae* (Shemi et al., 2015).

A)



B)

ATG core proteins	Yeast *S.cerevisiae*	Plants *A.thaliana*	Mammals
ATG1/ULK1 complex	ATG1 ATG13 ATG17 ATG29 ATG31 ATG11 --	ATG1A-ATG1C ATG13A,B -- -- -- ATG11 ATG101	ULK1, ULK2 ATG13 -- -- -- FIP200 ATG101
ATG9 and its cycling system	ATG9 ATG2 ATG18 ATG21	ATG9 ATG2 ATG18A-ATG18H --	ATG9A,B ATG2A,B WIPI1-WIPI4 --
Class III phosphoinositide 3 -kinase (PI3K) complex I	VPS30 ATG14 VPS15 VPS34 ATG38	ATG6 ATG14A,B VPS15 VPS34 --	Beclin1,2 ATG14 VPS15 VPS34 NRBF2
ATG12-ATG5 conjugation system	ATG12 ATG7 ATG10 ATG5 ATG16	ATG12A,B ATG7 ATG10 ATG5 ATG16	ATG12 ATG7 ATG10 ATG5 ATG16L
ATG8 conjugation system	ATG4 ATG7 ATG3 ATG8	ATG4A,B ATG7 ATG3 ATG8A-ATG8I	ATG4A-ATG4D ATG7 ATG3 LC3A-LC3C, GABARAP, GABARAPL1-3

Figure 2. Autophagy machinery in yeast, plants, and mammals. (A) Schematic of the different steps involved in autophagosome biogenesis (B) Core proteins participating in autophagosome formation.

1.4.2A Phagophore initiation and nucleation

Eukaryotic cells adjust their basic metabolic processes depending on the available resources and external conditions. They sense these factors either directly or indirectly, through signals like hormones or nutrients. Integral to this regulation is the Target of rapamycin (TOR) complex, a serine/threonine kinase complex, which under nutrient-rich conditions, upregulates protein translation and cell growth, and negatively regulates autophagy (Burkart & Brandizzi, 2021). In plants, TOR phosphorylates ATG13 under nutrient-rich conditions, thereby preventing its association with ATG1 (Son et al., 2018). Nutrient starvation reduces TOR activity, enabling ATG13 dephosphorylation and interaction with ATG1. This leads to the formation and activation of the ATG1 complex at multiple phosphatidylinositol 3-phosphate (PI3P)-enriched ER domains called phagophore assembly sites (PAS) (Zhuang et al., 2018) (Figure 3B). Subsequently, ATG1 complex recruits GA-derived vesicles containing the transmembrane ATG9 protein, which serve as the initial membrane source for phagophore nucleation (Zhuang et al., 2017). The ATG1 complex also recruits the PI3K complex, which produces PI3P essential for the downstream assembly of the autophagy machinery (Wun et al., 2020).

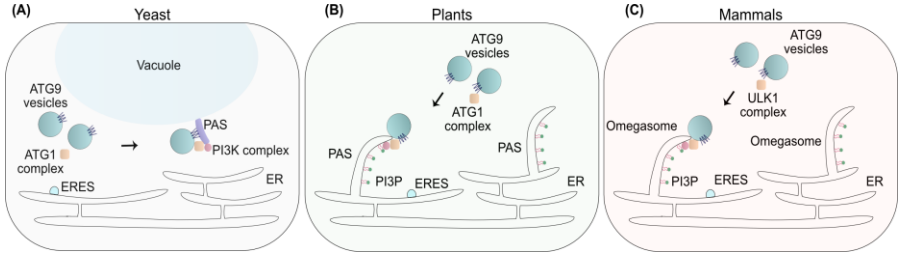


Figure 3. Schematic of the phagophore initiation step in yeast, plants, and mammals. (A) In yeast, the phagophore initiation occurs at a single Phagophore Assembly Site (PAS), which is in close proximity to the ER and vacuole. In plants (B) and mammals (C), phagophore initiation can occur at multiple phosphatidylinositol 3-phosphate (PI3P) enriched membrane structures on the ER.

In mammals, the ULK1 complex is constitutively formed, and TOR modulates its activity through phosphorylation of ULK1 and ATG13 (Y. Hu & Reggiori, 2022). Similar to plants, phagophore initiation in mammals occurs at multiple PI3P-enriched 'omegasomes' derived from the ER (Axe et al., 2008) (Figure 3C). In contrast, yeast has a single punctate PAS located in close proximity to the ER and vacuole (Itakura & Mizushima, 2010)

(Figure 3A). Presumably, these differences in the phagophore initiation evolved in response to divergent autophagy demands between yeast and the multicellular organisms.
It is important to note that during selective autophagy (explained further), initiation of the phagophore is TOR-independent. Instead, the ATG1/ULK1 complex is recruited to the cargo by the cargo receptors, via ATG11/ focal adhesion kinase family interacting protein of 200 kD (FIP200) (F. Li & Vierstra, 2014; Z. Zhou et al., 2021). Assembly of this complex at the cargo leads to its activation via autophosphorylation, subsequently recruiting the rest of the autophagy machinery to the site (Turco et al., 2020).

1.4.2B Phagophore expansion

Traditionally, during phagophore expansion, PI3P and ATG9 present on the phagophores recruit the binding proteins: ATG2-ATG18/WD-repeat protein Interacting with PhosphoInositides (WIPI) complex. In yeast, ATG2 binds to ATG9 and PI3P on the phagophore membrane, and facilitates ATG18 binding to the lipids (Gómez-Sánchez et al., 2018; Kotani et al., 2018), whereas in mammals, ATG2 is recruited after WIPIs bind to PI3P (Maeda et al., 2019). In plants, a recent study shows the plausible involvement of ATG18 in recruiting ATG2 and ATG9 to the phagophore membrane (Luo et al., 2023). Of note, as discussed previously, ATG2 proteins contain the lipid transfer Chorein-N domain. Consistent with this, ATG2 in yeast and mammals has been shown to be involved in transferring lipids from the ER to the phagophore, aiding its expansion (Osawa et al., 2019; Valverde et al., 2019). The phagophore expansion further relies on two Ubl conjugation systems- ATG12 and ATG8. These two systems are explained in detail in chapter 1.4.3.

1.4.2C Phagophore curvature

The process by which autophagosomes are formed involves distinct changes in the membrane, starting from punctate structures to ultimately forming a spherical vesicle with a double membrane. The question that follows is: what drives the phagophore curvature, and what determines the size of the autophagosomes? Although little is known about this in plants, insights from yeast and mammals demonstrate that the ATG12-5 complex, in association with ATG8-PE, forms a mesh-like structure around the artificial vesicles, which could potentially establish a structural framework for shaping the phagophore (Jensen et al., 2022; Kaufmann et al., 2014). This is further

supported by studies showing the involvement of lipidated ATG8 in spherical vesicle formation (Maruyama et al., 2021), and its amounts determining the size of the autophagosomes (Xie et al., 2008). Actin filaments in mammals also contribute to membrane shaping by scaffolding on the concave side of the phagophore mediated by the actin-capping protein, CapZ (Mi et al., 2015). In Paper I, we observe actin colocalizing with the autophagosome marker in the vacuoles of roots and shoots, under autophagy-inducing conditions. This could be a plausible indication of actin's akin function in plants.

1.4.2D Autophagosome maturation and fusion

After the phagophore bends into a spherical shape, the subsequent step involves pore closure, mediated by the endosomal sorting complexes required for transport (ESCRT) (Zeng et al., 2023; F. Zhou et al., 2019). Sealing of phagophores was previously considered extremely crucial since unsealed autophagosomes cannot fuse with the lytic compartment. However, a recent study in yeast demonstrates that unsealed autophagosomes can enter the vacuoles upon prolonged autophagy induction (Wu et al., 2022). It remains to be determined whether this adaptation is exclusive to yeast or if it can also occur in plants and mammals.

Upon completion, autophagosomes proceed to a maturation phase during which ATG8 proteins present on the outer membrane are removed by the action of the ATG4 protease. This step has long been recognized as pivotal and evolutionarily conserved (Nair et al., 2012). However, our findings presented in Paper II challenge this notion in plants, where delipidation of ATG8 from the outer membrane is dispensable for subsequent fusion. This discovery highlights a significant departure from the established understanding and emphasizes the uniqueness of the autophagic process in plants.

In yeast, autophagosomes are formed near the vacuoles, while in plants and mammals, they need to be transported to the lytic compartment (Figure 3). This transportation in mammals is facilitated by microtubules, actin filaments, and motor proteins, which act as "railroads" to traffic the vesicles to their final destination (Kast & Dominguez, 2017). However, in plants, there is currently no evidence of cytoskeletal proteins involved in the transport of autophagosomes.

The final fusion process with the lytic compartment begins with the tethering step. While the mechanism driving autophagosome fusion with vacuoles

remains unclear in plants, in yeast and mammals, it is facilitated by the homotypic vacuole fusion and protein sorting (HOPS) tethering complex. HOPS bridges the autophagosomes with the lytic compartment, priming them for fusion. This tethering event enables the assembly of specific SNARE (soluble N-ethylmaleimide-sensitive factor attachment receptor) protein complexes to form between the two compartments, which draws them close together, driving the fusion event (Gómez-Sánchez et al., 2021; Jahn et al., 2003). Fusion occurs between the outer membrane of the autophagosomes and the membrane of the lytic compartment. Thereafter, the inner membrane along with the cargo undergoes degradation by resident vacuolar/lysosomal hydrolases, and the resulting molecules are subsequently recycled back into the cytoplasm. In mammals, two intriguing processes have been identified to occur at this final step: following the lysosomal fusion with the autophagosomes, the autophagosomal membrane components such as ATG9 are recycled from autolysosomes via a process called autophagosomal components recycling (ACR) (C. Zhou et al., 2022). Subsequently, the lysosomal membrane proteins are recycled via a process called autophagic lysosome reformation (ALR) to regenerate functional lysosomes (Y. Chen & Yu, 2018).

1.4.2E Recycling

Autophagy plays a major role in metabolism by providing building blocks that can be utilized for essential anabolic synthesis. The degradation of proteins, lipids, carbohydrates, and nucleic acids results in the cytoplasmic release of amino acids, fatty acids, nucleosides, and sugars (Rabinowitz & White, 2010). In addition to the macromolecules, the cells also degrade and recycle specific organelles based on the prevailing conditions and the metabolic status of the cell. For example, in plants, Rubisco-containing bodies (RCBs) are degraded during carbon starvation, which provides free amino acids that can ultimately be used for ATP synthesis (Hirota et al., 2018). Similarly, lipid droplets are degraded under this condition to release fatty acids as an energy source (Fan et al., 2019).

1.4.3 The ubiquitin-like conjugation systems

1.4.3A ATG12 conjugation system

The ATG12-5/16 is a multimeric Ubl conjugation system that aids in the expansion of the phagophores. Assembly of the complex begins with

activation of Ubl ATG12 in an ATP-dependent manner by the E1-like ATG7, where its C-terminal glycine forms a thioester bond with the active site cysteine of ATG7. ATG12 is then transferred to the E2-like ATG10 and finally gets conjugated via its glycine residue to lysine 128 of ATG5 via an isopeptide bond (Matsushita et al., 2007; Mizushima, 2020). Subsequently, two sets of ATG12-ATG5 conjugates bind non-covalently with the dimeric ATG16, to form E3-like ATG12-ATG5/ATG16 complex.
In yeast and mammals, PI3P binding proteins, ATG21/WIPI2 recruit the ATG12-5 complex to the PAS (Juris et al., 2015; Strong et al., 2021). In yeast, it can also be recruited via the interaction of ATG12 with the ATG1 kinase complex (Harada et al., 2019). It remains unclear how this complex is recruited to PAS in plants. Nevertheless, at the PAS, the ATG12-5 complex exerts its E3-like activity by promoting the lipid conjugation of ATG8 (Fujioka et al., 2008; Hanada et al., 2007). Although dispensable for ATG8 lipidation, ATG16 determines the ATG8 lipidation site in mammals (Fujita et al., 2008; Hanada et al., 2007).

1.4.3B ATG8 conjugation system

ATG4

ATG4 is an evolutionarily conserved cysteine protease that regulates autophagy through the processing and deconjugating of ATG8. While yeasts have only one ATG4, plants and mammals possess two (ATG4A and ATG4B) and four isoforms (ATG4A-D), respectively. In mammals, ATG4B is considered to have the broadest range of activity (M. Li et al., 2011), while in plants, ATG4A is most active against all ATG8s, in vitro (Woo et al., 2014). Before participating in autophagosome biogenesis, ATG8 proteins are cleaved by ATG4 enabling its conjugation with phosphatidylethanolamine (PE) on the phagophore membrane. Additionally, during autophagosome maturation, ATG4 is responsible for cleaving ATG8 from the outer membrane of the autophagosomes. In yeast and mammals, a conserved ATG8 recognition site is identified in the C-terminus of ATG4, called cLIR (C-terminal LC3-Interacting Region) motif, which allows its binding to lipidated and unlipidated form of ATG8 (Abreu et al., 2017; Skytte Rasmussen et al., 2017). Additionally, in yeast, another motif called APEAR (ATG8-PE association region) specifically recognizes lipidated ATG8, facilitating the deconjugation of ATG8 from the outer membrane of the autophagosomes (Abreu et al., 2017). The dissociation of ATG8 from the

surface of autophagosomes necessitates checkpoints to prevent its premature removal by ATG4. This role is undertaken by the ATG1/ULK1 complex, which directly phosphorylates ATG4 proteins at the PAS, thereby regulating its function, and allowing for the complete formation of autophagosomes (Sánchez-Wandelmer et al., 2017).

ATG8

Central to the phagophore expansion and autophagosome formation is the Ubl ATG8. While yeasts have only one ATG8, mammals have six ATG8 homologs, which are further divided into two subfamilies based on their amino acid sequence similarity- Light Chain 3 (LC3) and Gamma-aminobutyric acid receptor-associated protein (GABARAP)/ Golgi-associated ATPase Enhancer (GATE-16) (Weidberg et al., 2010). As indicated by their nomenclature, these proteins were initially recognized for their autophagy-unrelated functions. For instance, GABARAPs were identified to participate in the transportation of transmembrane receptors from the GA to the PM (Leil et al., 2004), and subsequently as ATG8s, thereby emphasizing the broader involvement of key players of autophagy in other cellular processes. In the plant kingdom, ATG8 proteins are classified into 2 clades by phylogenetic analysis- Clade-I, where the members are closely related to fungi, and Clade-II, where they are more similar to mammals. *Arabidopsis thaliana* ATG8 encodes 9 isoforms, of which ATG8A-G belong to Clade I. ATG8H and ATGI, which do not have a C-terminal extension after glycine residue, are classified into Clade-II (Kellner et al., 2017). The structure of ATG8 proteins is conserved in eukaryotes, with the N-terminal helical domain formed by two α-helices, and the C-terminal ubiquitin domain containing the β-grasp fold (Shpilka et al., 2011). ATG8, as mentioned previously, is involved in phagophore expansion and autophagosome maturation. Additionally, ATG8 is also responsible for cargo recognition during selective autophagy. Selectivity is achieved by receptor-mediated binding of cargoes to the AIM (ATG8 interacting motif) in plants and yeast, equivalent to LC3 interacting region (LIR) in mammals. The core AIM sequence is defined as WXXL, an aromatic amino acid, followed by two random amino acids and an aliphatic amino acid (Noda et al., 2010). AIM binds with ATG8 on its hydrophobic patch called ADS/LDS (AIM- docking site/LIR-docking site). Notably, a unique interacting motif has been characterized in plants, called UIM (Ubiquitin-interacting motif), which docks onto the UDS (UIM-docking site) (Marshall et al., 2015). Since

ATG8 proteins are actively involved in autophagy and decorate the inner and outer membranes of the autophagosomes, they serve as excellent markers for studying autophagy.

ATG8 lipidation and delipidation

ATG8 proteins undergo posttranslational modifications before they can participate in autophagosome biogenesis. To enable the conjugation of ATG8 to PE, ATG4 cleaves ATG8 at its C-terminal, exposing a glycine residue. Of note, ATG8H and ATG8I in plants do not require this processing step (Yoshimoto et al., 2004). The exposed glycine forms a thioester bond with the E1-like ATG7, in an ATP-dependent manner. Activated ATG8 is then transferred to the E2-like ATG3 enzyme, via a thioester bond. In the final step, ATG8 gets conjugated to the headgroup of PE on the inner and outer membrane of the autophagosome, and this step is promoted by the E3-like ATG12-5/16 complex (Mizushima, 2020). The lipidated ATG8 further leads to membrane expansion (Nakatogawa et al., 2007). Besides processing ATG8 precursors, ATG4 is also responsible for deconjugating ATG8 from the outer membrane of the autophagosomes, where it cleaves the amide bond between ATG8 and PE, thereby releasing ATG8 back into the cytoplasm.

1.4.4 Substrates of autophagy

Autophagy can be non-selective or selective. Non-selective, bulk degradation is often described as the mechanism wherein the growing phagophores sequester a wide range of cytoplasmic material randomly. The bulk degradation process is TOR-dependent and occurs in response to starvation conditions (Y. Hu & Reggiori, 2022; Marshall & Vierstra, 2018). It is crucial for maintaining the cellular supply of lipids, amino acids, and nucleotides. Selective autophagy, on the other hand, relies on targeting and degradation of specific cellular components, including organelles and protein aggregates (Gatica et al., 2018; Stephani & Dagdas, 2020). It is mediated via selective autophagy receptors (SAR), which act as molecular bridges between the cargo and phagophores (Johansen & Lamark, 2011). SARs recognize cargo and facilitate the recruitment of the autophagy machinery to it, thereby closely aligning the cargo with the phagophore membrane. The process involves interaction with ATG8 present on the growing phagophores, via the AIM/LIR motifs. Selective autophagy can degrade invasive microbes (xenophagy) in mammals and plants (Hofius et al., 2017),

as well as damaged or superfluous organelles in eukaryotes, including the ER (reticulophagy), peroxisomes (pexophagy), chloroplasts (chlorophagy), ribosomes (ribophagy), proteosomes (proteophagy), aggregated proteins (aggrephagy), and mitochondria (mitophagy) (Marshall & Vierstra, 2018). In Paper I, we show that autophagy inherently involves a degree of quasi-selectivity. This selectivity is likely essential to ensure that cells do not 'randomly' degrade vital organelles or cellular components that are necessary for their proper functioning during starvation.

1.5 Adaptation and diversification of autophagy across kingdoms

Unicellular and multicellular organisms, with their own unique environmental conditions and ecological niches, require autophagy to adapt and meet these specific demands. Stepping back from the molecular level, let us understand how autophagy operates within the broader context of the organisms.

1.5.1 Autophagy in yeasts

Yeasts are unicellular organisms, which require the ability to swiftly adjust to shifting surroundings. One of the major challenges they face is the quality and quantity of nutrients. Autophagy is employed substantially during nitrogen and carbon starvation conditions (Abeliovich & Klionsky, 2001), indicating the adoption of this machinery as an alternate source of energy substrate in dire times. Moreover, in response to the environmental conditions, yeast can adjust their growth rate by altering the length of the cell cycle. They undergo rapid mitotic growth under nutrient-rich conditions. When the conditions are harsh, the diploid cells switch to meiosis and sporulation, which germinate under favourable conditions. A study shows that sporulation is arrested in autophagy-deficient mutants, indicating a potential cross-talk between the two pathways (Kuma & Mizushima, 2010). Unique to yeast is the non-induced autophagy, called cytoplasm to vacuole targeting (cvt) pathway. This pathway is active under nutrient-rich conditions in vegetatively growing yeast cells. It is a selective autophagy process that is involved in transporting vacuolar aminopeptidase 1 (Ape1) and aspartyl aminopeptidase 4 (Ape4) to the vacuole, where they mature and can serve enzymatic functions (Lynch-Day & Klionsky, 2010).

1.5.2 Autophagy in mammals

Multicellular organisms have evolved to comprise of different cell types which have their own metabolic and physiological needs. Autophagy has potentially taken over as a multifunctional pathway in higher eukaryotes to sustain these requirements. Evidently so, autophagy plays very important cell-type specific roles in mammals. Neurons, for example, are long-lived cells that rely on autophagy for their maintenance. Major neurodegenerative disease like Alzheimer's and Huntington's, which are characterized by accumulation of protein aggregates and damaged mitochondria is attributed largely to dysfunctional autophagy in the cells (Boland et al., 2018). On the other hand, the proliferation of hepatocytes (liver cells) during liver regeneration relies heavily on autophagy for its source of glucose, amino acids, and free fatty acids (Xu et al., 2020). Autophagy has also evolved tissue-specific functions, for example, it is involved in degrading the inhibitors of adipocyte differentiation and also maintaining homeostasis in adipose tissues (Y. Zhang et al., 2012). Apart from its beneficial functions, autophagy can also be detrimental to the organism, which is why it is often referred to as a double-edged sword. For instance, in cancer, autophagy initially serves to remove damaged or mutated cellular components, preventing the accumulation of harmful mutations. However, in established tumours, cancer cells can hijack the autophagy process to their advantage. They may use autophagy to survive and thrive in stressful conditions within the tumour microenvironment, such as nutrient deprivation and low oxygen levels. This can render cancer cells more resistant to therapies like chemotherapy and radiation, allowing them to continue growing and spreading (Chavez-Dominguez et al., 2020). Autophagy is also closely linked to apoptosis, where it can act as a pro-survival mechanism by delaying or preventing apoptosis by removing damaged components and providing energy during times of stress. These pathways also crosstalk, for example, where Beclin-2 can inhibit apoptosis and upregulate autophagy, depending on the conditions (Mukhopadhyay et al., 2014). Additionally, autophagy can switch to cell death during excessive or uncontrolled autophagy (Gozuacik & Kimchi, 2007). The large number of SARs identified in mammals highlights the multifaceted roles of autophagy in multicellular organisms (Kirkin & Rogov, 2019).

1.5.3 Autophagy in plants

Plants are sessile organisms, representing a unique form of multicellular life. They possess both above-ground autotrophic and below-ground heterotrophic organs. This dichotomy implies that they live in two different environmental conditions simultaneously, wherein, the above-ground autotrophic shoots are exposed to day/night conditions, while the below-ground roots are in constant darkness. The photosynthetic shoots serve as the carbon source for plants. Carbon fixed during the day is exported to the non-photosynthetic sink organs, such as roots and seeds, during the night (Durand et al., 2018). This results in what is called the source-sink relationship. Similarly, roots are responsible for absorbing water and minerals ions including nitrate from the soil, thus serving as the primary source. Nitrate is subsequently remobilized to the shoots where it is assimilated into amino acids (Masclaux-Daubresse et al., 2010), among other nitrogen-containing molecules. Plants, during their course of life, have to face a myriad of biotic and abiotic stress, including drought, lack of essential nutrients, temperature fluctuations, pathogens, and viruses. The spatio-functional division of plants enables them to respond to and partially overcome these challenges. For example, when faced with nitrogen-depleted conditions, shoots reallocate carbon assimilates to roots, thus allowing root foraging in order to acquire more nitrogen (Masclaux-Daubresse et al., 2010). Moreover, since chloroplasts store around 80% of leaf nitrogen, during senescence, the stromal proteins are degraded and the released nitrogen is remobilized. Autophagy has adapted to play a role here, where it can target and degrade chloroplasts via a process called chlorophagy, allowing for efficient nutrient remobilization (Nakamura & Izumi, 2018; Sakuraba, 2022). Autophagy also participates in regulating senescence, wherein autophagy-deficient mutants are shown to exhibit premature aging and early senescence (Minina et al., 2018).

Given the distinct physiological functions of these two organs and the unique metabolic needs of different cell types, it is reasonable to assume that the autophagy machinery has evolved to cater to these organ-specific demands. In Paper I, we have revealed the organ-specific responses to various autophagy-inducing conditions and the distinct subcellular level activity within these organs. In addition, we have identified cell type specificity of autophagy in Paper V.

1.6 Cross-talk between autophagy and UPS

Recent advancements have revealed that UPS and autophagy, once thought to be independent pathways, are indeed strongly intertwined (Ji & Kwon, 2017; Minina et al., 2017; Nag et al., 2023; Raffeiner et al., 2023). As elucidated in the previous chapters, Ubl proteins and Ubl conjugation systems are integral to autophagy. Aside from this, "actual" ubiquitination of key components of autophagy is required to regulate their stability. In plants, RING-finger E3 ligases SEVEN IN ABSENTIA OF *ARABIDOPSIS THALIANA* (SINAT) and the signaling adaptor E3 ligase TUMOR NECROSIS FACTOR RECEPTOR ASSOCIATED FACTOR (TRAF) controls the stability and dynamics of ATG1, ATG13 and ATG6. Under nutrient-rich conditions, SINAT1 and SINAT2, aided by TRAF1a and TRAF1b, target ATG6 and ATG13 for degradation, thereby restraining autophagy. During acute conditions, SINAT1 and SINAT2 drive the proteasomal degradation of ATG13, to moderate the autophagy intensity. During recovery, they target ATG13 for degradation, to terminate autophagy. In contrast, SINAT6 promotes autophagy during nutrient deprivation by interacting with ATG6 and ATG13, hindering their ubiquitination and degradation (Qi et al., 2017, 2020). In mammals, ULK1 and Beclin-1 are degraded by the 26S proteasome (Nazio et al., 2013; Shi & Kehrl, 2010). In yeast, there is evidence suggesting proteasomal degradation of ATG9 (G. Hu et al., 2020) and ATG32, albeit controversial (Y. Zhou et al., 2022). Ubiquitination is additionally required for selective autophagy, mediated by Ub binding cargo receptors, and Ub tags on the cargo as a cue for degradation (Marshall & Vierstra, 2018; Rogov et al., 2014).

Like they say, the hunter becomes hunted. Proteasomes in plants, under certain conditions, can be degraded by autophagy, via a process called proteaphagy. The proteasomes are extensively ubiquitinated, which are recognized by 19S RP REGULATORY PARTICLE NON-ATPASE 10 (RPN10) receptors via its UIM, and delivers the proteosomes to the UDS on ATG8 (Marshall et al., 2015). Initially discovered in plants, proteaphagy has now been identified in yeasts and mammals (Cohen-Kaplan et al., 2016; Marshall et al., 2016).

Additionally, UPS and autophagy can also work together within cells to manage proteotoxic stress. This dual activation is particularly evident during ER-stress in plants and mammals (Raffeiner et al., 2023; C. Wang & Wang, 2015).

1.7 Autophagy and other trafficking pathways

While autophagy is a fundamental pathway of the endomembrane trafficking system, it is important to note that other pathways, such as exocytosis and endocytosis, also play vital roles in maintaining the proper functioning of a cell (Figure 4).

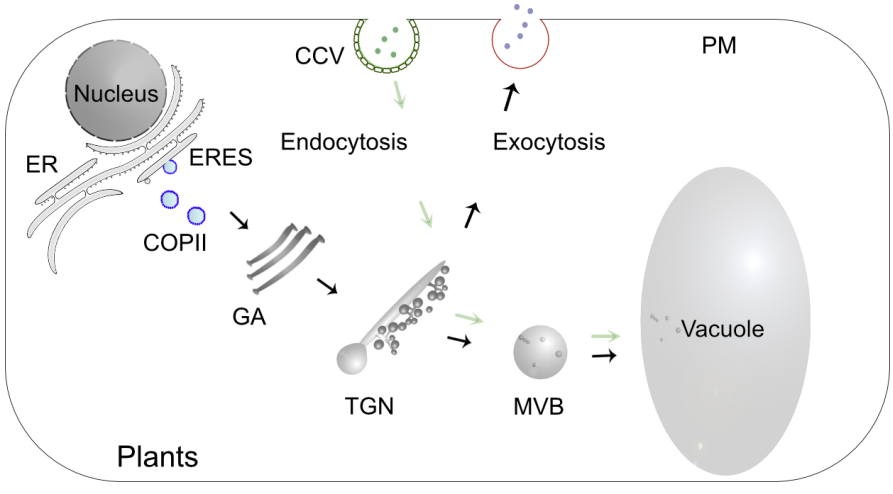


Figure 4. Schematic of exocytosis and endocytosis in plant cells. Proteins and lipids synthesized in the ER are transported to the GA by COPII vesicles, where they undergo processing and tagging. The processed molecules are trafficked to the TGN and are further exported to the PM via exocytosis, or trafficked to the vacuole via MVBs. PM proteins can also be endocytosed via Clathrin-coated vesicles (CCVs) to the TGN.

In exocytosis, the process begins with the anterograde trafficking of proteins and lipids synthesized in the ER to the GA, via ERES (and ERGIC in mammals). This is mediated by Coat protein complex II (COPII) vesicles. In the GA, these proteins and lipids undergo processing and are further transported to the TGN, which is the sorting and distribution hub of the system. From TGN, the molecules are either exported to the PM, or directed to the lytic compartment for degradation via the MVBs (Robinson et al., 2007; Rothman & Orci, 1992).

Endocytosis on the other hand involves the internalization of ubiquitinated PM proteins into the cell via clathrin-coated vesicles (CCV) and their transport to the TGN (Grones et al., 2022). Thereafter, they are sorted into the MVBs, to be delivered to the vacuole.

Multiple examples from the previous chapters indicate a strong interlink between autophagy and components of the endomembrane system. I will delve into this here, by unwrapping how ATGs transcend their well-defined roles, as well as the contributions of other trafficking systems to autophagy.

1.7.1 Role of ATGs beyond autophagy

In mammals, there is extensive data of ATGs and their non-canonical roles (Boya et al., 2013). To list a few, Vacuolar Protein Sorting 34 (VPS34), VPS15 and Beclin1 participate in endocytosis, while ATG4B, ATG8, ATG7 and ATG5 take part in exocytosis (Galluzzi & Green, 2019; Münz, 2021). In plants, a recent study has identified the role of ATG8 in heat stress response, where it is translocated to the swollen GA under these conditions, and recruits the clathrin component Clathrin light chain 2 (CLC2) to repair the damage (J. Zhou et al., 2023). Apart from this, non-lipidated ATG8 binds to ABNORMAL SHOOT 3 (ABS3) protein to control senescence under normal and nutrient-deprived conditions (Jia et al., 2019). In Paper III, the interactome of ATG5 also suggests for its role beyond autophagy.

1.7.2 Non-ATG regulators of autophagy

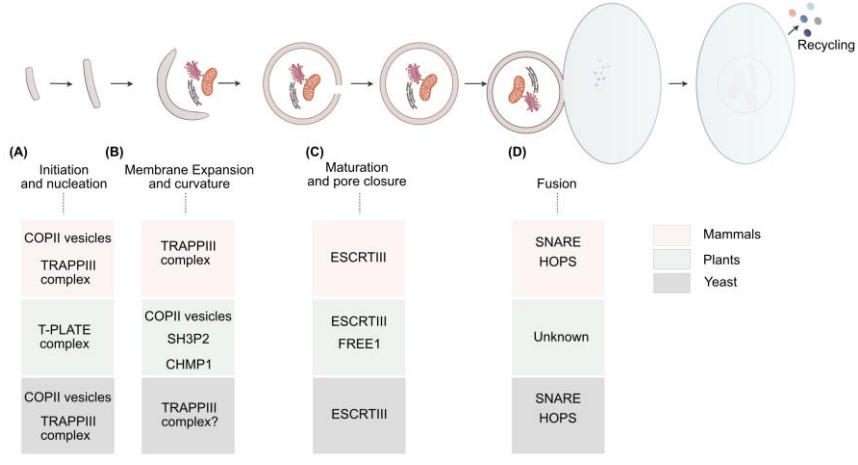


Figure 5. Components of the endomembrane trafficking pathways involved in the different steps of autophagosome biogenesis in yeast, plants, and mammals.

As mentioned previously, during starvation, autophagosome initiation occurs on the ER subdomains or in close proximity to the ER, which serves

as a lipid source for the growing phagophores. COPII vesicles derived from the ER possibly contribute to this by recruiting ER material as a membrane source. In plants, PI3P-associated FYVE2 proteins interact with a member of the COPII complex, Secretion-associated Ras-related GTPase 1 (SAR1). FYVE2 and SAR1 are recruited to the PAS during phagophore expansion (Kim et al., 2022) (Figure 5B). In yeast and mammals, COPII vesicles are recruited to the PAS during the nucleation step (Ge et al., 2014; Lemus et al., 2016; J. Wang et al., 2013) (Figure 5A). This recruitment is mediated by the Transport Protein Particle III (TRAPPIII), a conserved guanine nucleotide exchange factor (GEF) (Tan et al., 2013), which is also responsible for the trafficking of ATG9 to the PAS (Lamb et al., 2016; Shirahama-Noda et al., 2013). In mammals, TRAPPIII complex additionally recruits the ATG2-WIPI complex to the phagophore membrane during its expansion (Stanga et al., 2019). In plants, the role of TRAPP complexes in autophagy is undefined. Interaction of ATG5 with different TRAPP complexes might suggest its involvement in autophagy (Paper III)

The evolutionarily conserved ESCRT complexes, essential for sorting membrane cargo, and formation of MVBs (Gao et al., 2017; Henne et al., 2011), also play an important role in autophagy. In plants, there are three ESCRT complexes, (ESCRT I-III), whereas yeasts and mammals have four complexes each (ESCRT 0-III) (Winter & Hauser, 2006). Plants compensate for the absence of ESCRT 0 with a plant-specific FYVE DOMAIN PROTEIN REQUIRED FOR ENDOSOMAL SORTING 1 (FREE1) protein (Gao et al., 2014). A recent study has revealed the role of FREE1 in phagophore closure (Zeng et al., 2023). In yeasts and mammals, this function is fulfilled by ESCRT III (Takahashi et al., 2018; F. Zhou et al., 2019) (Figure 5C). Additionally, in plants, SH3 DOMAIN-CONTAINING PROTEIN 2 (SH3P2), which functions together with ESCRT I, localizes on the phagophores by interacting with PI3P, and is involved in phagophore expansion (Zhuang et al., 2013). Additionally, an ESCRT III subunit-CHARGED MULTIVESICULAR BODY PROTEIN1 (CHMP1), helps sequester plastids into autophagosomes, by promoting the phagophore expansion (Spitzer et al., 2015) (Figure 5B).

The phagophores can assemble at different membrane contact sites (MCS) (Zwilling & Reggiori, 2022), which are sites where membranes are juxtaposed. Autophagosome biogenesis is shown to occur at multiple ER-driven MCS, including ER-GA, ER-Mitochondria, and ER-PM (Ye et al.,

2020). Biogenesis occurring at the ER-PM contact site is aided by components involved in exocytosis and endocytosis. In plants, endocytosis, specifically clathrin-mediated endocytosis (CME), involves adaptor protein complexes- Adaptor Protein-2 (AP-2) and TPLATE complex (TPC) (Grones et al., 2022). One of the TPC members, AtEH/Pan1, regulates actin-dependent autophagy through its interaction with the actin nucleation protein, Arp2/3 (P. Wang et al., 2019). In mammals, WHAMM, a nucleation-promoting factor (NPF), aids in recruiting and activating the Arp2/3 complex to facilitate actin assembly at the autophagosome formation site (Kast & Dominguez, 2015). Furthermore, in mammals, the ER-localised protein, synaptotagmins, involved in tethering ER to the PM, is also involved in autophagosome biogenesis (Nascimbeni et al., 2017).

Since complexity is biology's way, endomembrane organelles that contribute to autophagy are also targeted by the catabolic process, depending on the prevailing conditions (Paper I). Evidenced by the critical roles these components play in the cell, it once again underscores the vital importance of cells precisely targeting their cargoes through a methodical approach.

1.8 Tools for studying autophagy in *Arabidopsis thaliana*

Research on autophagy in plants is an emerging field, with ongoing efforts aimed at gaining a deeper understanding of the underlying mechanisms. A majority of this research is carried out in *Arabidopsis thaliana*, recognized as a model plant due to its relatively small and completely sequenced genome. Arabidopsis can be easily manipulated through genetic engineering, efficiently transformed, and offers the advantage of a short life-cycle, allowing for easier repetition of experiments (Z. J. Chen et al., 2004). Our research group mainly focuses on the study of autophagy in Arabidopsis seedlings. These seedlings are typically grown in petri plates, enabling access to a large number of replicates. In studies relevant to this thesis, for example, studying the impact of nitrogen scarcity at the organ level and its resulting phenotypes, seedlings are grown in petri plates containing nitrogen-deprived medium. Similarly, to investigate the effects of carbon depletion, seedlings are grown on sucrose-deprived media and subjected to continuous darkness. To assess the impact of these depleted conditions on seedling phenotypes, we capture images at specific time intervals. However, manual imaging of these plates poses significant challenges, particularly during the

night, risking the omission of critical time-dependent data. Additionally, removing plates from the dark for imaging for the latter experiment carries the risk of unintended alterations to the data. To tackle these issues, we have developed SPIRO, an automated petri plate imaging robot (Paper IV).

One of the favored approaches to studying autophagy at the sub-cellular level is to use seedlings expressing fluorescently labeled autophagy reporters, which can then be tracked using a confocal microscope (Klionsky et al., 2008). However, these seedlings are extremely sensitive and fragile, and even slight mechanical stress can induce autophagy. This situation often occurs when transferring seedlings from petri plates to microscopy slides before imaging, which may result in data artifacts. Another limitation of this method is its reliance on end-point readouts, which do not provide real-time information on the dynamic nature of autophagy. To address these limitations, we have developed RoPod, a non-invasive toolkit for Arabidopsis root imaging (Paper V).

2. Storyline of the thesis

Research on plants provides a unique advantage over mammalian studies, as it allows simultaneous physical access to the entire multicellular organism, including various cells, tissues, and organs. This stands in contrast to mammalian research, which often relies on isolated cell lines or specific tissues. In plants, with their coexistence of autotrophic and heterotrophic functions, we hypothesize that autophagy, while active in all cells, plays distinct roles depending on the organ's source or sink functions.

Building upon this, we embarked on a study to delve into the dynamics of autophagic activity in the roots and shoots of *Arabidopsis thaliana* under different conditions that induce bulk autophagy. Owing to the significant differences in the proteome of the two organs, we further aimed to acquire a comprehensive understanding of the specific cellular components that are subject to autophagic degradation in these organs. In light of the conventional characterization of bulk autophagy as a random sequestration process, we also sought to explore the potential existence of inherent selectivity by studying the fate of various organelles in the two organs. **(Paper I)**

Our observations revealed that the roots and shoots exhibit distinct autophagic responses, prompting us to investigate how these organ-specific autophagic features are generated. We hypothesized that the multimember ATG gene families, which may have diversified their functions to adapt to the specific requirements of different organs or conditions, could be one of the factors responsible for these differences. To explore this further, we focused on two core proteins of the autophagy machinery, ATG4 and ATG8, and made an intriguing discovery related to their plant-specific roles. **(Paper II)**

The ATG4 and ATG8 investigation unveiled that one of the fundamental steps in the autophagic pathway is not conserved in plants, suggesting significant modifications to the molecular machinery of autophagy. We aimed to deepen our understanding of the mechanisms driving these modifications by exploring the potential autophagy-unrelated functions of ATGs. This approach was motivated by the recognition that ATGs are not

exclusively involved in autophagy; some of them have been found to have roles in other cellular processes. Investigating these non-autophagic functions of ATGs would provide valuable insights into the broader context of ATG proteins and how their multifaceted roles might contribute to the modifications observed in plant autophagy. **(Paper III)**

We developed SPIRO to facilitate the integration of molecular and cell biology findings from the above studies to the physiological implications, as exemplified in Paper II. **(Paper IV)**

In order to monitor autophagy at a high temporal resolution, we developed the RoPod microscopy chambers. These chambers were instrumental for Paper I, enabling us to select the most relevant time points for autophagic activity in roots, which was subsequently used for studying activity in shoots. **(Paper V)**

3. Discussion and conclusions

3.1 Plant-specific spatiotemporal dynamics of autophagy

The possession of autotrophic and heterotrophic organs within plants, and the ability to adjust their cellular processes in response to environmental changes, renders them an interesting system for investigating autophagy. This aspect of plant biology becomes even more apparent when considering the distinct metabolic processes occurring in shoots and roots, which may naturally lead to differing autophagic responses, as each organ has its unique nutritional and energy needs. Consistent with this, we present the first empirical evidence of time-resolved dynamics in autophagic activity in the two organs under different autophagy-inducing conditions. This is corroborated by the distinct spatiotemporal selectivity towards cargo, strongly suggesting that plant autophagy is tailored to uphold organ-specific functions. Additionally, we have acquired supporting evidence showing a certain level of selectivity in the process of bulk autophagy.

Previous studies have shown that Target of Rapamycin (TOR) is instrumental in maintaining growth and metabolic homeostasis. In response to abiotic stresses, TOR signaling is inhibited, thereby redirecting cellular resources away from metabolic pathways, toward stress responses (H. Zhang et al., 2020). Moreover, studies have shown that inhibition of TOR activity can result in the induction of bulk autophagy (Dong et al., 2015; Pu et al., 2017). In this work, we employed three different inhibitors of TOR activity to study the organ-specific response to bulk autophagy-inducing conditions, including carbon starvation (–C), nitrogen starvation (–N), and treatment with AZD8055 (AZD). Our findings demonstrate that under all three conditions, roots show an earlier and more robust autophagic response in comparison to the shoots (Paper I, figure 2G). We hypothesize that this could be due to the source-sink relationship and their competition for nutrients. For instance, when the availability of carbon is limited, it is known that Arabidopsis roots tend to grow significantly shorter (van Gelderen et al., 2018). This is because the endogenous energy resources produced via photosynthesis are primarily allocated to promote hypocotyl growth, leaving fewer resources for root development (García-González et al., 2021).

Interestingly, in autophagy-deficient mutants, the roots of seedlings grown on –C medium are significantly shorter than the wild-type (Paper II, figure 3), while there is no significant difference in the hypocotyl length between them. It is plausible that upregulated autophagy in the wild-type roots supports growth under –C to some extent by upcycling proteins. Conversely, root growth is an adaptive strategy of plants under –N (López-Bucio et al., 2003). Consistent with this, we see stimulated root growth in wild-type seedlings, while the autophagy-deficient mutants have stagnated root growth (Paper II, figure 3). Increased autophagic activity which we observe in roots could be an adaptive strategy to support the energy-intensive process of root elongation, thus enabling nutrient acquisition. AZD on the other hand has been shown to impede the growth of both roots and shoots (Montané & Menand, 2013). Future scope lies in unravelling the underlying reasons for the variations in autophagic activity between the two organs under this treatment. As we sought to validate these microscopy-derived findings using the Green Fluorescent Protein (GFP)-cleavage assay, we made a perplexing observation: samples under –N showed an accumulation of the full-length fluorescently labelled ATG8. This was unexpected, as autophagy induction typically leads to the cytoplasmic marker being delivered to the vacuole, where the fluorescent tag is cleaved, therefore leading to a decrease in the full-length protein, as observed with AZD and –C (Figure 6). Moreover, –N exhibited a weaker GFP cleavage in comparison to AZD and –C, despite the microscopy experiments clearly demonstrating a comparable presence of GFP-containing autophagic bodies in the vacuole.

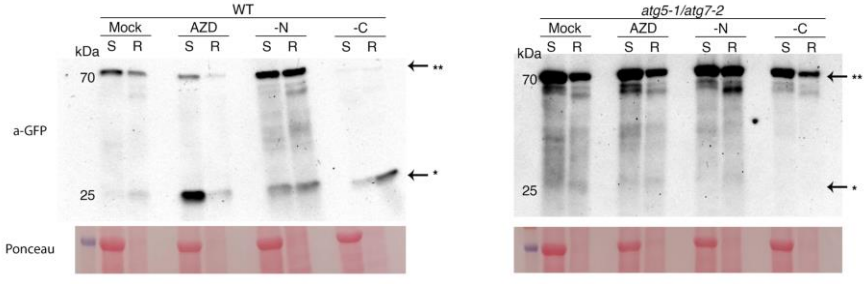


Figure 6. Accumulation of full-length pHusion proteins in the roots and shoots of wild-type Arabidopsis subjected to nitrogen starvation. In comparison to the seedlings subjected to AZD and –C, very little GFP-cleavage is observed in both organs under –N, which contradicts the microscopy data. Instead, there is a clear accumulation of the full-length pHusion proteins. **- pHusion-ATG8, *-Free GFP.

Having established the presence of organ-specific autophagic activity, we wanted to identify the targets of degradation in the two organs. To accomplish this, we performed mass spectrometry on vacuoles isolated from AZD-treated roots and shoots. The proteomics data revealed a quantitatively higher accumulation of proteins in the root vacuoles (Paper I, figure 5), thus corroborating the organ-specific activity. We subsequently selected the most interesting and physiologically relevant hits from both organs (Paper I, figure 5C), which present significant potential for future research. Of particular interest among the identified proteins were nuclear components that appeared to be targeted by autophagy in both roots and shoots. Autophagy-mediated degradation of nuclear components (nucleophagy) is an interesting process that appears to selectively nibble on portions of the nucleus at a time. This nibbling is logical, as engulfment of the entire organelle would be detrimental to the cells. However, there are some exceptions to this, for example, multinucleated filamentous fungi, where the entire nucleus is degraded to provide energy to the cells (Shoji et al., 2010). Nucleophagy is a relatively better-studied process in yeast and mammals, while there is currently no evidence of it occurring in plants. In yeast, degradation of the nuclear components occurs either via macronucleophagy or micronucleophagy, with the latter including piecemeal nucleophagy (PMN) and late nucleophagy (LN). PMN occurs at the nucleus vacuole junction under nutrient-rich conditions or short periods of –N, and its cargoes include nuclear envelope components and nucleolar proteins (Roberts et al., 2003). Contrarily, LN occurs when yeast cells undergo a prolonged period of starvation, delivering nucleoplasm components to the vacuole (Mijaljica et al., 2012). On the other hand, macronucleophagy is mediated by the yeast-exclusive ATG39 cargo receptors, which localize to the perinuclear ER, inner and outer nuclear membrane, and the cargo. ATG39 interacts with ATG8 via the ATG8 Interacting Motif (AIM), and the interaction results in recruitment of nuclear components to the vacuole at ATG11-positive sites (Mochida et al., 2015). This process therefore relies on the core autophagy machinery and is accountable for the partial degradation of a variety of nuclear components, including nucleolar proteins, nuclear envelope components, components of the nuclear pore complex (NPC), and even the NPC as a whole (C.-W. Lee et al., 2020; Yin & Klionsky, 2020). In mammals, the mechanisms underlying macronucleophagy are still elusive, and the initiators and receptors have not yet been identified. However,

evidence suggests a potential link between this process and pathological conditions, such as cancer and neurodegeneration (Sakuma & D'Angelo, 2017; Simon & Rout, 2014). Some of the nucleophagic substrates identified in mammals are the nuclear lamina components such as lamins (Dou et al., 2015) (absent in yeast), as well as the histone family proteins (Park et al., 2009; Rello-Varona et al., 2012). Our proteomics data revealed many nuclear proteins, including histone H2B protein as a potential substrate of autophagy in the roots and shoots. We therefore decided to empirically check for their targeting using transgenic lines expressing H2B-GFP, under AZD treatment. Unfortunately, this marker line did not reveal any evidence of nucleophagy in either of the organs (data not shown). It is worth noting that our microscopy experiments were limited to the epidermal cells of the roots and shoots, while the mass-spectrometry samples contained cells derived from the entire organs. This leaves open the possibility that nucleophagy occurs in other, less accessible cell types. However, since there is no concrete evidence of nucleophagy occurring in mammals and plants, it could also be possible that this process has evaded these organisms altogether, and what we see is circumstantial or a basal turnover of the nuclear proteins. Nevertheless, it would still be valuable to check the other nuclear proteins that have been identified in our study.

In the vacuolar proteome, we also found cytoplasmic ribosomal proteins along with various organellar proteins, which are indicative of SAR (Selective autophagy receptor)-independent and SAR-dependent targeting, respectively. Does this mean that there is simultaneous sequestration of these cargo-types into the same autophagosome during bulk autophagy? In answer to this, our Transmission Electron Microscopy (TEM) (Paper I, figure 3E) data yielded compelling evidence showing autophagic bodies (Abs) that consist solely of ribosomes or organelles, indicative of passive diffusion or active uptake of cargo into the autophagosome, respectively. Additionally, we also observed Abs containing ribosomes along with different organelles, implying co-occurring uptake of cargo. Our microscopy experiments further support this data, where we observe the simultaneous uptake of receptors and non-native cytoplasmic proteins (Paper I, figure 3A, C). In order to understand if there is a discernible pattern of selectivity influenced by the degree and type of stress in the two organs, we selected several organelle markers co-expressing the autophagosomal marker. Interestingly, we discovered that different organelles were either temporally targeted as the

treatment progressed, or selectively protected during the entire period. Moreover, the selectivity or protection of these organelles also varied between the two organs. Based on the chronological uptake or protection, we classified them into three groups: immediate uptake, delayed uptake, and no uptake (Paper I, figures 4 and S7). Intriguingly, as gathered from the –C and –N experiments, the grouping of the organelles also varied depending on the type of stress (Paper I, figures S8 and S9). These observations conceivably defy the definition of bulk autophagy, which is characterized as random receptor-independent sequestration of cytoplasmic material into the autophagosomes (Zaffagnini & Martens, 2016). We have thus revised the understanding of bulk autophagy to encompass a certain degree of selectivity. The findings of this paper raise several open questions. Firstly, why is there a spatiotemporal selectivity towards some organelles? One possible explanation is that the cells protect or sacrifice organelles based on their importance to the organ, depending on the trigger. For example, mitochondria in roots and shoots are known to have different proteomes, which results in their organ-specific roles (C. P. Lee et al., 2011). In our –C experiments, we see mitochondria as an immediate target in the roots, while being selectively protected in the shoots. Under this condition, mitophagy in the roots may help upcycle the protein reserves, supporting growth, while shoot mitochondria are protected to undergo metabolic readjustments and serve as alternative source of respiratory substrates (Law et al., 2018). An alternative explanation lies in the cells' ability to regulate the size and number of organelles under stress (Hickey et al., 2023). For example, peroxisomes proliferate to accommodate various metabolic demands or as a stress response. However, since excess organelles can be costly to maintain, cells use autophagy to eliminate them when conditions improve, in order to maintain cellular homeostasis (Olmedilla & Sandalio, 2019). Based on this notion, it is likely that some of the other organelles in our study also undergo temporal proliferation or restructuring in order to meet the organ's demands, which are later eliminated by autophagy (Bernales et al., 2006; Palikaras & Tavernarakis, 2014). Understanding this selectivity further will give us insights into organelle prioritization during stress and cellular adaptation. These answers may extend to broader cell biology principles, shedding light on organelle dynamics, resource allocation, and adaptation strategies. It would be worthwhile to investigate this in the future by quantifying the organelles in the cytoplasm before and through the period of stress, which

will additionally provide us with the intensity of uptake of each of the organelles over time. Another question we are prompted to ask is- does the TEM evidence, which shows certain organelles coexisting, or exclusively present within Abs, suggest a SAR-based specificity for organelles contained within these Abs? Addressing this question is crucial for understanding the selectivity mechanisms governing bulk autophagy. Further, it is unlikely that the nine ATG8 isoforms in Arabidopsis have redundant functions. It would therefore be interesting to explore the functional specialization of these isoforms and their potential cargo specificity.

In summary, this study has unveiled plant-specific adaptations of autophagy. It has provided insights into the spatiotemporal dynamics of autophagy in both source and sink organs under various stress conditions, shedding light on how plants allocate resources and devise growth strategies. The discovery of selective protection and targeting of different organelles during bulk autophagy underscores the crucial roles these organelles play in maintaining organ function.

3.2 Delipidation of ATG8 is not fundamental for plant autophagy

ATG8 proteins are key players in autophagy which decorate the inner and outer membranes of the autophagosomes. To achieve this, ATG8 undergoes post-translational modifications, where ATG4 cleaves their C-terminus to expose a glycine residue. Subsequently, the processed ATG8 proteins are attached to phosphatidylethanolamine (PE) on the phagophore membranes. This step is reversible, as ATG4 delipidates ATG8 from the outer membrane of the autophagosomes. Previously, it was believed that these evolutionarily conserved steps were fundamental in autophagy. However, our research demonstrates that although lipidation of ATG8 is important, its delipidation is dispensable for the normal functioning of autophagy in *Arabidopsis thaliana*, while still holding relevance in the green algae, *Chlamydomonas reinhardtii*.

Previous understanding of the importance of ATG8 delipidation in plants comes from studies performed by Yoshimoto, K. et al, where they observed that GFP-ATG8I, when overexpressed in ATG4-deficient plants, is not delivered to the vacuole under -N (Yoshimoto et al., 2004). This was attributed to the impairment in ATG8I delipidation from the outer membrane

of the autophagosomes. The read-out for this experiment was the absence of autophagosomes in the cytoplasm, and green haze in the vacuole. Although GFP is used extensively in autophagy-related studies, it is important to note that this protein is not stable in the acidic vacuoles due to its degradation by the vacuolar proteases (Tamura et al., 2003), unless an inhibitor of the vacuolar proton pump is used. This read-out could be the major limitation in their study, as we provide evidence of GFP-ATGI containing autophagic bodies in the vacuoles of ATG4 deficient plants (Paper II, figure 4C, F). Of note, the intensity of activity observed here is reduced in comparison to the wildtype plants, which could be another contributing factor for Yoshimoto, K. et al. to conclude otherwise (Paper II, figure 4G). In addition to ATG8I, we also demonstrate the efficient delivery of artificially truncated ATG8E to the vacuoles in ATG4 deficient plants, which further confirms that delipidation of ATG8 is not required for the optimal functioning of autophagy in Arabidopsis (Paper II, figure 1D). While this step is dispensable, the impairment of autophagy in ATG5 and ATG7 mutants confirms that lipidation of ATG8 is crucial in plants (Paper II, figure 4, S6). Contrarily in mammals, mutants of ATG3 and ATG5 only delay the phagophore expansion, but do not abolish autophagosome formation (Tsuboyama et al., 2016). A corroborating study shows formation of autophagosomes in the absence of ATG8, albeit smaller and with lesser efficiency (Nguyen et al., 2016). In addition, the former study demonstrates the essentiality of ATG8 lipidation for degrading the inner-autophagosomal membrane after fusing with the lysosome, while the latter shows the importance of ATG8 in autophagosome-lysosome fusion. If ATG8 lipidation is dispensable in mammals, and if ATG8-containing autophagosomes are still able to fuse with the lysosomes, what is the need for delipidation of ATG8, if there is one? In addition, could the non-essentiality of ATG8 lipidation be a reason for the lipidation-independent functions of ATG5 in other cellular processes (Baines et al., 2022)?

In summary, this study challenges the conventional understanding that the core autophagy step of ATG8 delipidation is highly conserved and crucial among eukaryotes. It highlights the functional specialization of Arabidopsis ATG8 isoforms in autophagosome formation. These findings not only provide a new perspective on plant-specific aspects of autophagy but also underscore the diversity and adaptability of autophagic mechanisms across

different species, challenging the notion of uniformity in this essential cellular process.

3.3 Autophagy (in)dependent roles of ATG5

Organisms often evolve by repurposing existing cellular machinery to serve new functions. Identifying the dispensability of ATG8 delipidation in plants led us to contemplate if the core components of the autophagy machinery have taken on additional roles independent of autophagy. In answer to this, we present the interactome of Arabidopsis ATG5, containing the regulators of autophagy along with stress-response factors, potential partners of the nuclear fraction of ATG5, components of the UPS system, as well as the endomembrane trafficking system. In addition, we have identified Post Translational Modifications (PTMs) present on the ATG12-5 complex.
In this study, we identified proteins pulled-down with wildtype ATG5, which is capable of forming a complete ATG5-ATG12/ATG16 complex that is required for autophagy. We compared it with proteins pulled-down with the mutant ATG5K128R, which can only form the minimal ATG5-ATG16 complex (Paper III, figure 1A). Of particular interest from the list of interactors (Paper III, figure 2C and D) were the nuclear proteins. In mammals, ATG8 undergoes deacetylation in the nucleus by the nuclear deacetylase Sirtuin1, which enables its subsequent translocation to the cytoplasm, during starvation (Huang et al., 2015; I. H. Lee et al., 2008). Sirtuin1 has additionally been shown to deacetylate ATG5 and ATG7 to initiate autophagy (I. H. Lee et al., 2008). Based on these studies, we speculate that plant ATG5 might undergo deacetylation within the nucleus as part of its regulatory mechanism, before being transported to the cytoplasm. This hypothesis is supported by the localization of ATG8 and ATG5 in the plant nucleus (Paper III, figure 3A), as well as the identification of ATG5 acetylation during basal autophagy. Additionally, the identification of nuclear proteins involved in photomorphogenesis defines the plant-specific functions of ATG5. The impaired germination rate of ATG5 mutants in the absence of nitrogen and light (Paper IV, figure 5) provides strong initial evidence for the potential role of ATG5 in photomorphogenesis.

This study unveils the potential multifaceted roles of ATG5 in plants, serving additional functions independent of autophagy. The identification of post-translational modifications on the ATG12-5 complex adds depth to our

understanding of its regulatory mechanisms. The potential role of ATG5 in photomorphogenesis opens up avenues for future research exploring the role of autophagy in plant development.

4. Future research

Science is an ongoing endeavor, never truly complete. The discovery of interesting and novel aspects of autophagy in plants has not only expanded our understanding but also paved the way for new research avenues to explore. If presented with the opportunity to build upon the findings of this thesis, I would pursue the following, in addition to investigating the questions posed in chapter 3.

In paper I, we show that roots and shoots exhibit lower autophagic activity under –N conditions. This is corroborated by the low-intensity delayed uptake, or selective protection of several organelles under this condition in both organs. These findings imply that autophagy under –N is highly selective towards its targets. If this is indeed the case, and considering that the autophagy-deficient mutants have significantly shorter roots in comparison to the wild-type, the question remains as to what are the targets of degradation under this condition. To answer this question, TEM can be a valuable tool. Firstly, it can help us determine whether autophagy is indeed highly selective under –N. If we observe a substantial percentage of autophagosomes tightly enveloping individual organelles, a characteristic feature of selective autophagy, it will confirm our hypothesis. Secondly, besides ER which we have identified as a target under this condition, TEM can aid in identifying other organelles present within the autophagic bodies (Abs). To further support these findings, MS can be employed to identify any additional cargo that may remain undetected by TEM. Additionally, in paper II, we observed that overexpressing ATG8I results in shorter root growth under –N. This perplexing finding may suggest that ATG8I acts as a toggle switch, playing specific roles in regulating the autophagy activity under this condition. Generating ATG8I knockouts and analysing their phenotypes can provide further insights into this. In addition to organ-specific activity, it will be interesting to build upon cell-type autophagy specificity under different conditions. Presumably, each cell-type adopts autophagy to cater to its distinct physiological and metabolic requirements, as evidenced by the difference in autophagic activity in trichoblasts and atrichoblasts (paper V, Figure 5). Cell-type autophagy specificity can further mean that there are differences in selectivity at the sub-cellular level. Since our work was restricted to studying sub-cellular activity in the epidermal cells only,

broadening this knowledge to other cell-types will be beneficial in understanding the contribution of different organelles in meeting the cells' requirements. This work can further be supported by generating transgenic lines co-expressing multiple organelle markers along with the autophagy reporter. In addition, it will be compelling to use organelle markers expressing other isoforms of ATG8 and compare the resulting data with our current work. This will help us better our understanding of the specificity of the isoforms in relation to cargo.

It has been shown that the upregulation of autophagy results in improved seed yield, plant growth, and longevity (Minina et al., 2018). In an era where global food security is under constant threat due to climate change and population growth, understanding the role of autophagy in crop plants becomes even more critical. By investing in further research to deepen our understanding of the molecular mechanisms of autophagy and its impact on plant growth and resilience, we can develop targeted strategies to enhance crop yields and nutritional content. This research, in the long run, will have the potential to contribute significantly to global food security, helping us meet the nutritional needs of a growing population. Additionally, since nitrogen limitation is a common challenge faced by field crops, building upon our understanding of autophagy under this condition in Arabidopsis will prove valuable, and the knowledge gained can further be transferred to food crops to enhance their resilience.

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Popular science summary

Autophagy, akin to the concept of sustainability in our daily lives, is a vital cellular process that ensures the efficient recycling and reuse of resources within cells of all eukaryotes. Just as sustainability emphasizes responsible consumption, waste reduction, and resource conservation for the well-being of our planet, autophagy plays a similar role at the cellular level, safeguarding the health and longevity of the cells. Consider cells as miniature ecosystems, constantly generating "waste", or cargo, in the form of damaged organelles, misfolded proteins, and other cellular debris during their regular activities. Autophagy serves as the cellular sustainability program, undertaking the collection, recycling, and repurposing of this cargo. The process is mediated by double-membraned structures called autophagosomes which collect the cargo from the cytoplasm and deliver it to the recycling station, called vacuole, where it is degraded by resident enzymes, and repurposed. Autophagy can be of two types- bulk and selective. Bulk autophagy refers to the indiscriminate clearing of cytoplasmic material, while selective autophagy specifically targets its cargo for degradation.

Plants are unique organisms that possess both autotrophic and heterotrophic organs which are exposed to different environmental conditions. The above-ground autotrophic organs, like leaves, conduct photosynthesis to produce energy from sunlight, while the below-ground heterotrophic organs, such as roots, absorb water and nutrients from the soil to support plant growth. This duality in their physiological functions results in the two organs having different metabolism. This brings us back to autophagy, which plays a crucial role in metabolism, wherein, the recycled materials are inputs for metabolism, through which they generate energy for sustaining growth and development. The question that follows is- does the differences in

metabolism in the two organs result in autophagy being regulated accordingly?

In this thesis, using the model plant organism *Arabidopsis thaliana*, we show the differential autophagic response of roots and shoots under various stress conditions, with the roots always undergoing an earlier and stronger autophagic activity. Additionally, we have discovered that bulk autophagy does not indiscriminately clear out cargo, instead, it involves specific targeting in both organs. Additionally, we have identified the specific cargo that is delivered to the vacuole, which opens up significant scope for research to understand how their recycling helps in the well-being of plants.

The formation of autophagosomes relies on AuTophaGy-related proteins or ATGs, with ATG8 playing a key role. For ATG8 to function in this process, it must be cleaved at its C-terminal end by a specific enzyme called ATG4. Following this cleavage, ATG8 gets attached to the lipids on the inner and outer membranes of the autophagosomes. Prior to these autophagosomes entering the vacuole, ATG8s are cleaved from its outer membrane by ATG4, via a process called delipidation. This step was previously considered fundamental in all eukaryotes, as inefficient delipidation was thought to inhibit autophagosome fusion with the vacuole, thereby disrupting the entire pathway. In this thesis, we provide evidence that, in plants specifically, the delipidation of ATG8 by ATG4 is not crucial for autophagy. In addition to this novel finding, we have gathered evidence of ATGs potentially playing additional roles beyond autophagy in other cellular processes.

To successfully carry out these studies, we developed an imaging robot called SPIRO, which allows for continuous imaging of the seedlings. This time-lapse imaging system proved crucial for comparing and studying the phenotypes of Arabidopsis seedlings grown on different nutrient mediums. We also developed specialized microscopy chambers called RoPod, which aided us in the continuous monitoring of autophagy in Arabidopsis roots.

In summary, through this thesis, we have unraveled the complexity and specificity of plant autophagy, paving the way for further investigations into the molecular mechanisms governing this process. Building upon these findings holds promise in enhancing our knowledge of how plants adapt to changing environments and can have future applications in improving crop resilience and yield.

Populärvetenskaplig sammanfattning

Autofagi, kan jämställas med konceptet hållbarhet i vårt dagliga liv och är en vital cellulär process som säkerställer effektiv återvinning och återanvändning av resurser inom celler hos alla eukaryoter. Precis som hållbarhet betonar ansvarsfull konsumtion, avfallshantering och resursbevarande för planetens välbefinnande, spelar autofagi en liknande roll på cellulär nivå och skyddar cellernas hälsa och livslängd. Man kan betrakta celler som miniatyr-ekosystem som konstant genererar "avfall" eller last i form av skadade organeller, felveckade proteiner och annat cellulärt skräp under sin regelbundna verksamhet. Autofagi fungerar som det cellulära hållbarhetssystemet och samlar, återvinner och återanvänder ”avfallet”. Processen medieras av en dubbelmembranstruktur som kallas autofagosomer, som samlar ”avfallet” från cytoplasman och levererar den till återvinningsstationen, kallad vakuol, där det bryts ned av lokala enzymer och återanvänds. Autofagi kan vara av två typer - bulk och selektiv. Bulkautofagi avser den ospecifika rensningen av cytoplasmatiskt material, medan selektiv autofagi riktar sig till specifikt ”avfall” för nedbrytning.

Växter är unika organismer som har både autotrofa och heterotrofa organ som utsätts för olika miljöförhållanden. De autotrofa organen ovan jord, som blad, utför fotosyntes för att producera energi från solljus, medan de heterotrofa organen under jorden, som rötter, absorberar vatten och näringsämnen från marken för att stödja växttillväxten. Denna dubbelhet i deras fysiologiska funktioner resulterar i att de två organen har olika ämnesomsättning. Detta tar oss tillbaka till autofagi, som spelar en avgörande roll i ämnesomsättningen, där de återvunna materialen är insatser för denna process, genom vilken de genererar energi för att stödja tillväxt och utveckling. Följdfrågan är om skillnaderna i ämnesomsättningen i de två organen resulterar i att autofagi regleras därefter? I den här avhandlingen

visar vi, med hjälp av modellväxtorganismen *Arabidopsis thaliana*, den differentierade autofagiska responsen hos rötter och skott under olika stressförhållanden, där rötterna alltid genomgår en tidigare och kraftigare autofagisk aktivitet. Dessutom har vi upptäckt att bulkautofagi inte urskillningslöst rensar cellmaterial utan inriktar sig specifikt på det i båda organen. Därtill har vi identifierat den specifika lasten som levereras till vakuolen, vilket öppnar upp betydande möjligheter för forskning i att förstå hur deras återvinning av material bidrar till växters välmående.

Bildandet av autofagosomer är beroende av autofagi-relaterade proteiner eller ATG:er, där ATG8 spelar en nyckelroll. För att ATG8 ska fungera i denna process måste det klyvas vid sin C-terminala ände av ett specifikt enzym som kallas ATG4. Efter denna klyvning fäster sig ATG8 vid lipider på autofagosomens inre och yttre membran. Innan dessa autofagosomer kommer in i vakuolen klyvs ATG8 från dess yttre membran av ATG4 genom en process som kallas delipidering. Det här steget ansågs tidigare vara grundläggande för alla eukaryoter, eftersom ineffektiv delipidering ansågs hämma fusionen av autofagosomer med vakuolen och därigenom störa hela reaktionsvägen. I den här avhandlingen presenterar vi bevis för att delipidering av ATG8 genom ATG4 inte är avgörande för autofagi i växter. Utöver detta nya resultat har vi samlat bevis på att ATG:er potentiellt spelar ytterligare roller utöver autofagi i andra cellulära processer.

För att framgångsrikt genomföra dessa studier utvecklade vi en bildrobot kallad SPIRO, som möjliggör automatiserad och kontinuerlig avbildning av växtskott. Systemet som tar bilder i regelbundna tidsintervall i både ljus och mörker var avgörande för att jämföra och studera fenotyperna hos Arabidopsis-skott som odlades på olika näringsmedier. Vi utvecklade även specialiserade mikroskopikammare kallade RoPod, som hjälpte oss att med minimal stresspåverkan kontinuerligt övervaka autofagi i Arabidopsis-rötter.

Sammanfattningsvis har vi genom denna avhandling avslöjat komplexiteten och specificiteten i växters autofagi, vilket banar väg för vidare undersökningar av de molekylära mekanismerna som styr denna process. Vilket röjer väg för att öka vår kunskap om hur växter anpassar sig till föränderliga miljöer och kan ha framtida tillämpningar som förbättrar grödors motståndskraft och avkastning.

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संजू, thank you! Jab bhi lage na ho paave, yeh dinn yaad karrna!

III

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Article

Interactome of *Arabidopsis* ATG5 Suggests Functions beyond Autophagy

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Abstract: Autophagy is a catabolic pathway capable of degrading cellular components ranging from individual molecules to organelles. Autophagy helps cells cope with stress by removing superfluous or hazardous material. In a previous work, we demonstrated that transcriptional upregulation of two autophagy-related genes, *ATG5* and *ATG7*, in *Arabidopsis thaliana* positively affected agronomically important traits: biomass, seed yield, tolerance to pathogens and oxidative stress. Although the occurrence of these traits correlated with enhanced autophagic activity, it is possible that autophagy-independent roles of ATG5 and ATG7 also contributed to the phenotypes. In this study, we employed affinity purification and LC-MS/MS to identify the interactome of wild-type ATG5 and its autophagy-inactive substitution mutant, ATG5^{K128R} Here we present the first interactome of plant ATG5, encompassing not only known autophagy regulators but also stress-response factors, components of the ubiquitin-proteasome system, proteins involved in endomembrane trafficking, and potential partners of the nuclear fraction of ATG5. Furthermore, we discovered post-translational modifications, such as phosphorylation and acetylation present on ATG5 complex components that are likely to play regulatory functions. These results strongly indicate that plant ATG5 complex proteins have roles beyond autophagy itself, opening avenues for further investigations on the complex roles of autophagy in plant growth and stress responses.

Keywords: plant proteomics; plant ubiquitin-like conjugation system; autophagy-unrelated functions; nuclear ATG5; nuclear ATG12; posttranslational modifications; PP2A; HXK1; endomembrane trafficking; proteasome

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1. Introduction

Plants must endure a range of unfavorable environmental conditions to survive and propagate. Heat, drought, water logging, salinity, and pests are but a few examples of environmental stresses, which plants must cope with. By utilizing autophagy, plant cells can dispose of harmful cellular constituents and recycle the material for other purposes. During macroautophagy (hereafter autophagy), cellular constituents are engulfed by a de novo formed double-membrane vesicle called autophagosome and delivered to the lytic vacuole for degradation [1]. The autophagy pathway is regulated by approximately 40 autophagy-related (ATG) proteins, which include about 20 core ATGs generally grouped into five

large complexes/systems essential for autophagic activity [2]. Central to the autophagic pathway is the lipidation of ATG8, which comprises a series of reactions enabled by ATG4, ATG7, ATG3, ATG10 and ATG5-ATG12/ATG16 complex culminating in the conjugation of ATG8 with phosphatidylethanolamine (PE). ATG8-PE is anchored on the membrane of the forming autophagosome facilitating the membrane elongation, cargo recognition and autophagosome trafficking [3,4].

In our previous study [5], we upregulated individually *ATG5* and *ATG7*, encoding for two core autophagy proteins required for the lipidation of ATG8. We discovered that upregulation of either of these two genes positively affected autophagic activity without impacting the transcription of the other genes participating in the ATG8-lipidation, indicating that ATG5 and ATG7 are rate-limiting components of the autophagic pathway. Interestingly, overexpression (OE) of these genes promoted plant growth, seed yield and longevity. Furthermore, OE plants developed more inflorescences and exhibited prolonged flowering time, thus producing more seeds. Furthermore, *ATG5* and *ATG7 OE* had a delayed onset of leaf senescence compared to wild-type (WT) plants and improved resistance to necrotrophic pathogens and oxidative stress [5].

Even though the observed phenotypic traits correlated with upregulated autophagic activity in the OE plants, we still could not decidedly claim that these traits were caused solely by upregulated autophagy. The question remained whether ATG5 and ATG7 might also act in other pathways.

Indeed, there are several examples of ATG proteins participating in autophagy-unrelated pathways. For instance, ATG8 orthologs in animal cells partake in intracellular trafficking and Golgi transport [6]. A plant ATG8 ortholog was shown to interact with ABNORMAL SHOOT3 at the late endosome to promote senescence by protein degradation, this interaction did not require ATG8 conjugation with PE [7]. Furthermore, a product of ATG5 proteolytic cleavage was shown to act as an apoptotic effector in animal cells [8].

Arabidopsis knockout (KO) mutants of core *ATG* genes show no discernible phenotypes at early developmental stages, when grown under standard conditions [5]. However, they undergo early senescence, display increased stress-susceptibility, and compromised immunity to necrotrophs compared to WT plants [5]. Interestingly, KO of different *ATG* genes display a range of phenotypes under autophagy-inducing stress conditions. For example, while *atg5* and *atg7* plants have similar phenotypes [5], *atg2* plants display more severe senescence and growth stagnation symptoms [9], whereas *atg9* plants have less severe symptoms [10]. Since autophagy is abrogated in all these mutants, the difference in the phenotypes might stem from autophagy-unrelated functions of the encoded ATG proteins, as we hypothesised for *ATG5* and *ATG7* OE plants.

To shed light on autophagy-unrelated functions of the ATG proteins, we began with identifying ATG5 interactome under standard growth conditions that do not boost autophagy. ATG5 is known to form complex with ATG12 and/or ATG16 [11,12]. In *Arabidopsis* cells most of ATG5 is covalently linked to ATG12 [5] via a C-terminal glycine residue of ATG12 bound to a side chain of a lysine residue of ATG5. An AlphaFold2-generated structure prediction for Arabidopsis ATG5 [13,14], revealed its structural similarities with the better characterized ortholog of ATG5 from yeast [11], enabling a prediction of Lys128 (K128) as the lysine residue binding to ATG12.

ATG16 in turn, binds non-covalently to ATG5 to form an ATG5-ATG12/ATG16 complex, which possesses E3-like ligase activity and is further referred to as "complete complex". ATG16 recruits the complete complex to the phagophore, where its E3-like ligase activity is implemented to conjugate ATG8 to the resident PE [15]. Interestingly, ATG5 and ATG16 can interact in the absence of ATG12 to form a different complex [12], which in this study is named "minimal complex".

Here, we identified proteins pulled-down with two types of bait: WT ATG5 capable of forming a complete ATG5-ATG12/ATG16 complex that is required for autophagy and the mutant $ATG5^{K128R}$ that can form only the minimal ATG5-ATG16 complex (Figure 1A). We demonstrated only partial overlap between autophagy-competent ATG5 and autophagy-

incompetent ATG5^{K128R} interactomes. This functional network unveils previously unknown interacting partners of plant ATG5 and suggests its autophagy-unrelated functions, thereby providing a new insight into diverse roles ATGs play in plant growth, development, and stress responses.

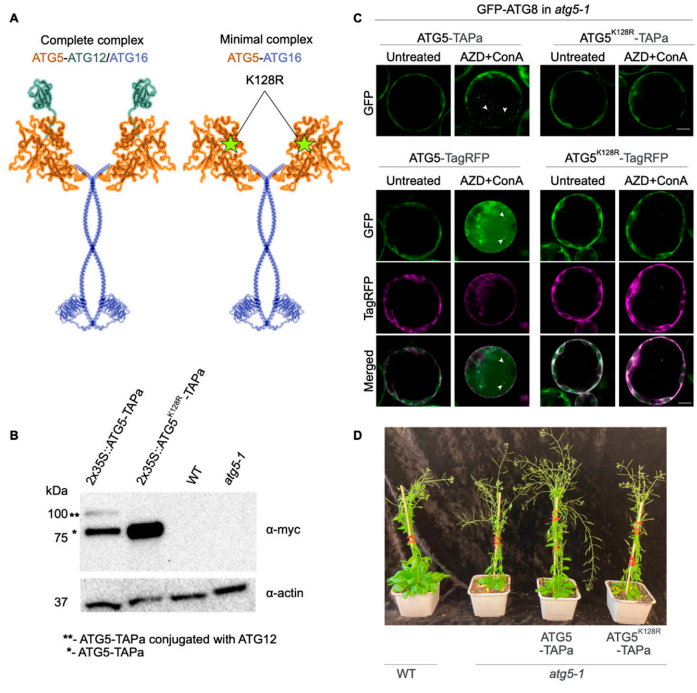

Figure 1. ATG5 K128R mutation abrogates conjugation to ATG12 and formation of the complete complex. (**A**). A schematic representation of the two complexes used to identify putative autophagy-unrelated interactors of ATG5: the complete complex encompassing ATG5-ATG12/ATG16 and a minimal complex comprising ATG5-ATG16. The K128R point mutation was predicted to impede covalent conjugation of ATG5 to ATG12 leading to the formation of only minimal ATG5-ATG16 complex. The complete complex is essential for autophagy, while the minimal complex is more likely to have autophagy-unrelated functions. (**B**). Western blot analysis demonstrating the absence of ATG5-ATG12 conjugation in Arabidopsis transgenic line expressing ATG5^{K128R} -TAP. α-actin was used for protein loading control. (**C**). The ATG5^{K128R} mutant fails to restore autophagy in *ATG5*-deficient cells. Confocal microscopy demonstrating accumulation of autophagic bodies (white arrowheads) in the vacuole of Arabidopsis protoplasts. Protoplasts were isolated from *atg5-1 Arabidopsis* plants expressing GFP-ATG8a and transformed with plasmids encoding ATG5-TAP, ATG5^{K128R}-TAP, ATG5-TagRFP, and ATG5^{K128R}-TagRFP. To induce autophagy, protoplasts were treated with 5μM AZD and 1 μM ConA for 24 h prior to imaging. Scale bars, 10 μm. (**D**). Representative pictures of two-month-old *Arabidopsis* plants under normal growth conditions (16 h 150 μM light, 22 °C). The ATG5-TAP fusion protein is expressed under the native ATG5 promoter complements the autophagy-deficient phenotype of the *atg5-1* mutant, unlike the ATG5^{K128R}-TAPa mutant.

2. Results

2.1. Generation and Characterization of Arabidopsis ATG5-TAP Lines

To identify interactors of both complete (ATG5-ATG12/ATG16) and minimal (ATG5-ATG16) complexes (Figure 1A), we engineered genetic constructs encoding for Tandem Affinity Purification (TAP) tag [16] fused with the C-terminus of ATG5, ATG5^{K128R} mutant or GFP, placed under the control of 2x35S promoter or *ATG5* native promoter. We predicted that ATG5^{K128R} mutation impairs the conjugation of *Arabidopsis* ATG5 to ATG12 at the early steps of the autophagy pathway (Figure 1A).

The resulting constructs were introduced into *Arabidopsis* Columbia-0 (Col-0) WT or autophagy deficient *atg5-1* background. Two transgenic lines per construct showing expression detectable by immunoblot were selected for further studies. To confirm that ATG5^{K128R} mutation indeed impairs conjugation with ATG12, formation of the complete complex and thus abrogates autophagy, we firstly performed immunodetection of the TAP tag in the transgenic lines expressing WT form of ATG5 or its K128R mutant. Indeed, the band corresponding to ATG5-TAP-ATG12 conjugate was not detectable in the protein extracts from plants expressing ATG5^{K128R} (Figure 1B).

We further verified that the K128R mutant is not able to restore autophagy in *ATG5*-deficient cells. For this, we isolated protoplasts from *atg5-1* plants expressing GFP-ATG8a marker for autophagosomes. Protoplasts were transformed with plasmids encoding TagRFP- or TAP-tagged ATG5 or ATG5^{K128R} and treated with AZD8055 and Concanamycin A (ConA) to induce autophagy and block degradation of autophagic bodies in the vacuole [17]. Upon induction of autophagy, GFP-positive autophagic bodies accumulated in the vacuoles in the presence of ATG5, but not in the presence of ATG5^{K128R}, corroborating the inability of this K128R mutant to form the functional complete complex required for autophagy (Figure 1C).

Finally, we assessed phenotypes of *atg5-1* plants complemented with ATG5-TAP or ATG5^{K128R}-TAP driven by the native *ATG5* promoter. In contrast to WT and ATG5-TAP complemented line, the ATG5^{K128R}-TAP complemented lines failed to rescue autophagy deficiency phenotype manifested in early senescence, smaller rosette leaves and reduced number of inflorescences (Figure 1D).

2.2. Interactomes of ATG5 and Its Complete and Minimal Complexes

2.2.1. Affinity Purification

The aforementioned *Arabidopsis* lines expressing ATG5-TAP, ATG5^{K128R}-TAP or GFP-TAP under control of 2x35S promoter in the WT (Col-0) background were used for the pull-down assay. Tandem affinity purification was performed as described earlier [18] on the protein extracts obtained from fully expanded leaves harvested at the bolting stage of plants grown under standard long day conditions. This developmental stage was previously shown to correlate with a low basal autophagy level [5] and was chosen to enrich for autophagy-independent interactors of ATG5. Pull-down for ATG5 and ATG5^{K128R} was performed in three biological replicates, each comprising a pool of more than 15 plants. The pull-down for the negative control (GFP-TAP) was performed in two biological replicates sampled in an identical manner. Purified proteins were analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and resulting data was analyses using MaxQuant and MSqRobSum [19].

2.2.2. Detection of Post-Translational Modifications

ATG proteins are constitutively expressed and undergo various post-translational modifications (PTMs) allowing rapid adjustment of their activity during switches between normal and stress conditions. For instance, inhibitory phosphorylation of mammalian ATG5 and ATG12 by ATG1 kinase governs spatio-temporal control of ATG8 lipidation during phagophore expansion [20], while inhibitory acetylation of mammalian ATG5, ATG7, ATG8 and ATG12 by the p300 acetyltransferase [21] is reversed by deacetylase Sirt1 during autophagy induction [22].

The role of PTMs in plant autophagy regulation is scarce and even less is known about the role of PTMs in the autophagy-unrelated functions of the plant ATG proteins. Therefore, we searched our LC-MS/MS data for the PTMs reliably detectable by mass spectrometry and also relevant for autophagy regulation, i.e., phosphorylation and acetylation, and detected PTMs of ATG5 and ATG12 (Figure 2A, Tables S1–S3). Interestingly, phosphorylation of Ser187 was identified only in ATG5, while acetylation of Lys183 was detectable on both ATG5 and ATG5^{K128R} indicating that the latter modification might also play a role in autophagy-independent function of ATG5. Additionally, phosphorylation of Ser7 was present at detectable levels on the ATG12A protein, indicating potential conservation of inhibitory phosphorylation of plant ATG12 under nutrient-rich conditions.

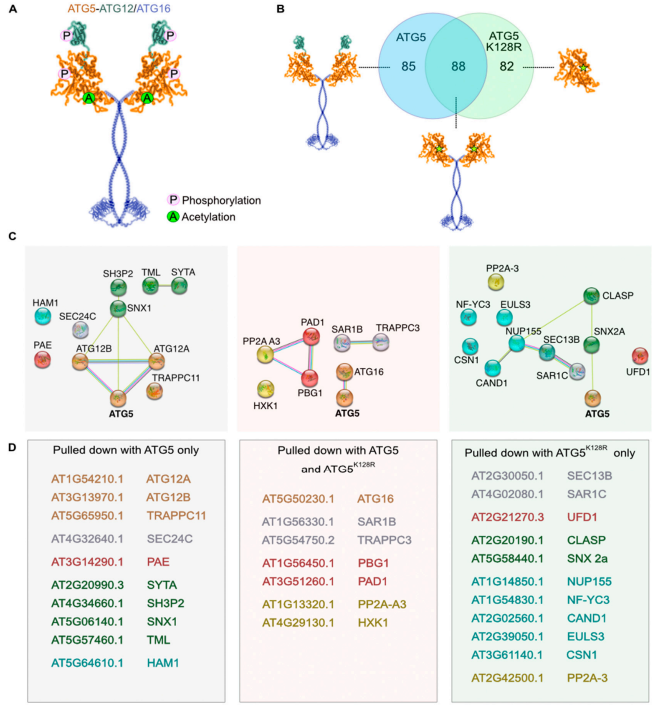

Figure 2. ATG5 and ATG5^{K128R} interactomes overlap only partially. (**A**). Post-translational modifications identified using LC-MS/MS. Phosphorylation of Lys183 was detected for both ATG5 and ATG5^{K128R}, while phosphorylation of Ser187 was detected for ATG5 only. Additionally, we observed phosphorylation of Ser7 for ATG12A. (**B**). Venn diagram displaying partially overlapping interactome networks of ATG5 and ATG5^{K128R}. Eighty-five proteins were found in ATG5 pull-down samples only, indicating that those might be interactors of either complete complex or of ATG12. Eighty-two proteins were pulled down only with ATG5^{K128R} indicating that they might be interacting with the individual ATG5 form, potentially the region masked in the ATG5-ATG12 conjugate. Eighty-eight proteins were shared between ATG5 and ATG5^{K128R} pull-downs, suggesting that those might be interactors of the minimal complex. (**C**). STRING analysis of the selected interactors pulled down with ATG5 only, ATG5^{K128R} only and with both types of baits. (**D**). List of selected proteins pulled down with ATG5 only, ATG5^{K128R} only and with both types of baits.

2.2.3. Criteria for Identifying ATG5 Interactomes

In order to identify specific and common interactors of the minimal and the complete complexes, we selected proteins enriched in ATG5^{K128R} and/or in ATG5 samples compared to the GFP-control. Significant interactors of ATG5/ATG5^{K128R} were identified using MSqRobSum (see Methods), requiring a minimal fold change of 1.5 (compared to GFP control) and p value < 0.05. After filtering, we retained 85 interactors only present in the ATG5 pull-down, 82 only in the ATG5^{K128R} pull-down and 88 common interactors in both ATG5 and ATG5^{K128R} pull-downs (Figure 2B). From here on, we grouped potential interactors into three categories: proteins pulled-down with ATG5 only (complete complex-specific), with ATG5^{K128R} only (potential interactors of individual ATG5) and with both ATG5 and ATG5^{K128R} (minimal complex-specific) (Figure 2C,D).

In accordance with conditions used for the affinity purification experiment, the typical autophagy-related interactors of ATG5, including ATG3, ATG10 and ATG8 [12], were not detectable in our samples, confirming that the identified candidate interactors were likely enriched for autophagy-unrelated pathways. Expectedly, ATG12A and ATG12B were only detected in the interactome of the complete complex, whereas ATG16 was detected in both interactomes (Figure 2C,D).

2.2.4. Putative Interactors Acting as Stress Sensors

Interestingly, we identified PP2A-A3, the scaffolding subunit of the Protein Phosphatase 2A (PP2A), as an interactor of the minimal complex, while a catalytic subunit of PP2A (PP2A-3) was pulled down with autophagy-incompetent ATG5^{K128R}. In mammalian cells PP2A is known to be an important regulator of the above mentioned PTMs, namely it dephosphorylates ATG13 upon inhibition of the TORC1 kinase complex enabling formation of ATG1-ATG13 complex and induction of autophagy [23]. Furthermore, different subunit compositions of the heterotrimeric PP2A have been shown to play alternative roles in animal autophagy [24]. Although plant PP2A is known to be important for abiotic and biotic stress response [25], its role in plant autophagy is still unknown and our observations provide the first insight on its potential participation and indicate a possible implication of autophagy-unrelated crosstalk between ATG5 and PP2A.

In addition to PP2A, we identified hexokinase 1 (HXK1, Table S3) as a potential interactor of the minimal complex. HXK1 was previously shown to suppress plant autophagy under glucose-rich conditions via an unknown mechanism [26]. Our finding suggests that HXK1 might not be acting via TORC1 as suggested previously but rather through the direct interaction with ATG5 and/or ATG16.

2.2.5. Putative Interactors Belonging to Endomembrane Trafficking System

Autophagy is an integral part of the endomembrane trafficking system [27,28] and so far, ATG8 has been the best characterized molecular link between plant autophagosomal structures and other components of the endomembrane trafficking system [29]. Remarkably, we discovered a large set of proteins playing a role in endomembrane trafficking that interact with ATG5 and its complete and minimal complexes: proteins involved in COPII-mediated endoplasmic reticulum (ER) to Golgi transport (SAR1B, SAR1C, SEC24C, TRAPPC3), phagophore formation (TRAPPC11, SH3P2), ER-PM contact sites (SYT1, TPLATE subunit TML), retromer complex subunits (SNX1 and SNX2a) and a CLASP protein involved in membrane loading of SNX1 (Figure 2C,D, Table S3). Most interestingly, our results showed that homologous proteins encoded by multi-member gene families, with not yet fully resolved redundancy, show specificity towards either the WT or autophagy incompetent ATG5 bait. For example, SAR1C was detectable in the ATG5^{K128R} pull-down only, unlike SAR1B that was found in both. Similarly, SNX1 was detected only in ATG5 pull-down samples, while SNX2a only in ATG5^{K128R} (Figure 2C,D, Table S3).

2.2.6. Putative Interactors Belonging to Ubiquitin-Proteasome System

Autophagy is tightly interlinked with another catabolic pathway, governed by the ubiquitin-proteasome system (UPS) [30,31]. Furthermore, mammalian ATG5 was shown to directly interact with UPS components to aid mitophagy [32]. Our affinity purification assay allowed detection of Ubiquitin Fusion Degradation 1 (UFD), a component of the CDC48 complex that was previously suggested to crosstalk with autophagy to help maintenance of chloroplastic proteins during oxidative stress [33] (Table S3). Furthermore, surprisingly, we discovered selective interaction between 20S proteasome subunit alpha E2 and complete ATG5 complex, while subunits alpha D1 and beta G1 showed preference towards the minimal complex (Table S3).

2.2.7. Putative Interactors of Nuclear-Localized ATG5 and ATG12

Interestingly, we also identified two components of nuclear pore complex as potential interactors of individual ATG5: Nucleoporin 155 (NUP155) and SEC13B (Figure 2D, Table S3) [34]. The mammalian ortholog of ATG5 has been shown to translocate to the nucleus under stress conditions and play a role in arresting cell division [35]. Furthermore, deacetylation-regulated nuclear export of a mammalian ATG8 ortholog was suggested to be implicated in autophagy [36]. In addition, plant ATG8s were previously observed in both cytoplasm and nuclei [5,17,37]. Therefore, we decided to investigate if the plant ATG5 complex components might also be localizing to the nuclei. For this we compared localization of ATG5, ATG8, ATG12 (A and B isoforms), and ATG16 fluorescent fusions transiently expressed in *Nicotiana benthamiana* epidermal leaf cells (Figure 3). To our surprise, ATG5 and both ATG12 isoforms, but not ATG16, could indeed localize to the nuclei under normal conditions, similarly to the previously observed localization of ATG8 (Figure 3A).

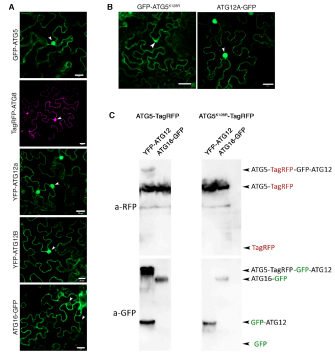

Figure 3. Conjugation is dispensable for nuclear localization of *Arabidopsis* ATG5 and ATG12. (**A**). Localization of fluorescently labelled ATG5, ATG8, ATG12A, ATG12B and ATG16 transiently expressed in *Nicotiana benthamiana* epidermal leaf cells. White arrowheads indicate nuclei position. Scale bar, 20 mm. (**B**). Localization of fluorescently labelled conjugation-incompetent protein forms of ATG5 and ATG12 transiently expressed in *N. benthamiana* epidermal leaf cells. Both, ATG5^{K128R} and ATG12-GFP are detectable in the nuclei, indicating that conjugation is dispensable for nuclear localization of these proteins. White arrowheads indicate nuclei position. Scale bar, 20 mm. (**C**). Western blot analysis of total protein extracts from *N. benthamiana* leaves expressing fluorescently labelled ATG5, ATG12 and ATG16 confirms formation of the covalent ATG5-ATG12 conjugate if the WT, but not K128R mutant of ATG5 is expressed, and shows no presence of the free fluorescent tag in the samples.

To further elucidate whether ATG5 and ATG12 localize to the nucleus in a form of the stable ATG5-ATG12 conjugate, we checked localization of the ATG5^{K128R} defective in conjugation to ATG12 and of the of ATG12A-GFP, in which GFP impedes conjugation with ATG5. We observed that ATG5^{K128R} and ATG12A-GFP still could be found in the

nuclei (Figure 3B), suggesting that conjugation is dispensable for nuclear localization of these proteins. These experiments were performed using GFP, YFP and TagRFP fusions of the proteins to ensure that observed localization was not an artefact caused by the fluorescent tag (only representative data for one of the fusion types is shown for each protein of interest). Finally, we used Western Blot analysis to exclude the possibility that nuclear signal observed by confocal microscopy resulted from passive diffusion of the fluorescent tag cleaved off the expressed fusion proteins. Detection of GFP and TagRFP in the protein extracts from leaves transiently expressing fluorescent fusions of ATG5, ATG12 and ATG16 confirmed the presence of intact fluorescent fusions (Figure 3C). In sum, these results demonstrated that at least three components of the autophagy ubiquitin-like conjugation system (ATG5, ATG12 and ATG8) localize to the nuclei. Furthermore, our affinity purification assay indicates that ATG5 might shuttle between nuclei and cytoplasm through interaction with the nuclear pore complex components NUP155 and SEC13b.

The molecular mechanisms underpinning typical autophagy-deficient plant phenotypes such as premature senescence and early onset and cessation of flowering have been only partially explained [9,38,39]. Our pull-down assay indicated interaction between ATG5 and nuclear proteins involved in stress response, flowering and photomorphogenesis (Table S3). Those include HAM1, a catalytic subunit of NuA4 acetyltransferase complex with a role in chromatin remodelling during environmental response of *Arabidopsis* [40], lectin EULS3 involved in osmotic stress response and ABA signalling [41] and Nuclear Factor YC protein 3 (NF-YC3) playing a role in photomorphogenesis, drought response, flowering and ABA signalling [42,43], COP9 signalosome subunit 1 involved in photomorphogenesis [44] and CULLIN-ASSOCIATED AND NEDDYLATION DISSOCIATED 1 (CAND1), a known mediator of auxin signaling and flowering [45].

3. Discussion

3.1. Evolutionary Context of Autophagy

Compartmentalization of biosynthesis and catabolism in eukaryotic cells lead to development of an intricate endomembrane trafficking system comprising a set of membrane-bound organelles, each having a specialized function in the multistep delivery of cellular materials to their respective destinations. For example, proteins synthesized on the ER can be delivered to the lytic compartment or the plasma membrane (PM) via stepwise transport through the Golgi apparatus and the endosomal system [46]. Similarly, degradation of the cellular content might also rely on directed trafficking towards a specialized compartment [46,47].

Autophagy and the ubiquitin-proteasome system (UPS) are the two major catabolic pathways in eukaryotic cells that counterbalance biosynthesis. UPS, which relies solely on protein-based molecular machinery for substrate recognition, labelling and degradation [48], is likely a more ancient catabolic process compared to the autophagic pathway. Unlike UPS, autophagy [48] is an integral part of the endomembrane trafficking system and requires formation of a membrane compartment (autophagosomes) to sequester cargo and deliver it for degradation to the lytic compartment, and thus would not be feasible in prokaryotic organisms lacking endomembrane compartments. Indeed, a prototype of the eukaryotic UPS-like system was discovered in *Archaea* [49], while autophagy is known to be typical for eukaryotic organisms [43].

A significant increase in cell structure complexity of eukaryotes created a necessity for a catabolic pathway able to degrade such cargoes as large as complete organelles. Autophagy was possibly established as such endomembrane trafficking-based high-throughput catabolic mechanism in the last eukaryotic common ancestor (LECA) [43], which already possessed a functioning UPS. Subsequently, although UPS and autophagy are often referred to as separate catabolic pathways, they might actually comprise two branches of a single proteolytic network in which autophagy was established after UPS. Co-evolution of both pathways sculpted intricate mechanisms of mutual regulation, implementing common molecular tools such as ubiquitin-like conjugation systems and overlapping degrons [50].

Since ATG proteins have also evolved as an integral part of the endomembrane trafficking system, it is not unlikely they might participate in the routes of the endomembrane trafficking that are not directly linked to autophagy, in its classical depiction. One such example is the cytoplasm-to-vacuole targeting(cvt) pathway in yeast. This is the only known biosynthetic pathway that utilizes autophagic machinery, where core ATG proteins, with a minor change in complex formation deliver enzymes to the vacuole [51]. Furthermore, the endomembrane systems of plant, animal and fungi have significant structural and functional differences, e.g., the lytic compartments are represented either by vacuoles or by lysosomes, the Golgi apparatus is either a static ribbon like structure or mobile stack or even individual cisternae, and the formation and function of endosomes varies significantly [52–55]. The evolution of autophagy as the integral part of such endomembrane systems likely diverged to develop kingdom-specific features. There are two pending questions. First, how did the evolution of the endomembrane system impact autophagy-unrelated functions of ATG proteins while preserving their role in autophagy and cross-talk with UPS? Second, did ATG proteins carry over some autophagy-unrelated functions from their distant homologs found in prokaryotes [43]?

3.2. Closely Related Components of Endomembrane Trafficking Pathways Show Selectivity towards Different ATG5 Baits

In this study we initiated a discovery proteomics approach to expand our understanding of plant ATG5 in autophagic pathway and beyond it. By comparing proteins pulled-down using WT or autophagy-incompetent K128R mutant of ATG5 as a bait we identified potential interactors of complete and minimal ATG5 complexes, and of individual ATG5.

Remarkably, we identified examples of close homologs presumably involved in the same pathway interacting with ATG5 but showing different preferences towards autophagy-competent or autophagy-incompetent forms of the bait. These results can be further developed into a new tool to elucidate specific functions of these homologs and bring important insights on the roles of ATG5 in and beyond autophagy.

For example, Secretion-Associated-Ras-related GTPase (SAR1) plays a crucial role in the initiation of COPII vesicles formation and thus enables ER to Golgi trafficking [56]. *Arabidopsis* genome encodes five paralogs of SAR1A-E with suggested tissue specific expression and at least partial functional diversity [57–59]. Only SAR1B and SAR1D were shown to play a role in autophagosome maturation [60] and autophagosome biogenesis [61], respectively. Our pull-down assay identified SAR1B as an interactor of the minimal ATG5 complex and SAR1C as an interactor of specifically $ATG5^{K128R}$. The latter interaction was most likely to be an example of autophagy-independent role of ATG5, as SAR1C was previously shown to not co-localize with autophagic structures under autophagy-inducing conditions [61].

Another example of such specificity towards the two ATG5 baits are sorting nexins (SNXs), subunits of a conserved among eukaryotes retromer complex, which is essential for retrograde trafficking and autophagy [62,63]. Elucidating the interactome of plant SNXs is especially interesting, as, despite being conserved, they seem to be dispensable for the functionality of the plant retromer complex [64] and might have evolved plant-specific functions instead [65]. In this study, we observed SNX1 being pulled-down only with the WT form of ATG5 (complete complex) and SNX2A only with the $ATG5^{K128R}$ (individual ATG5). Additionally, we also identified CLASP protein required for SNX1 membrane association [66] as a potential interactor of the $ATG5^{K128R}$. Future studies will help to elucidate the mechanism behind these intriguing specific preferences of SNX1 and SNX2A towards either WT or K128R mutant of ATG5, respectively, and potentially bring better understanding of the individual roles of SNXs.

Additionally, in this study, out of three existing *Arabidopsis* paralogs of SEC24 [57] which is a COPII coat complex component regulating vesicle formation during ER to Golgi trafficking, we identified only SEC24C as an interactor of complete ATG5 complex. Interestingly, the closest mammalian ortholog of SEC24C was shown to play a role in

selective autophagy of ER (ER-phagy) [67]. Future studies involving ER-phagy-inducing conditions will help to assess the potential role of SEC24C in plant ER-phagy and the role of ATG5 in it.

3.3. Cross-Talk of ATG5 and Clathrin-Mediated Trafficking Is Conserved

Lastly, we uncovered a set of ATG5 interactors that are associated with ER-PMCS (endoplasmic reticulum- plasma membrane contact sites) and clathrin-mediated vesicular trafficking (TPLATE COMPLEX MUNISCIN-LIKE (TML), SH3 DOMAIN-CONTAINING PROTEIN2 (SH3P2), and SYNAPTOTAGMIN 1 (SYT). All three proteins are known to partake in autophagy [68–70]. However, our study provides novel evidence of their interaction with the ATG5 complexes. Interestingly, a recent publication on the ATG5 interactome in mice [12] also presented several interactors of ATG5 related to clathrin-mediated trafficking, linking this pathway to animal autophagy.

4. Methods

4.1. Plasmids Construction

For generation of 2x35S:ATG5-TAP constructs, the ATG5 gene AT5G17290.1 without UTRs was amplified using PE7 and PE8 primers (Table S4) and cloned into pDONR/Zeo vector using Gateway cloning system (Invitrogen/Thermo Fisher Scientific, Waltham, MA USA). The point mutation was introduced into this entry clone using primers PE10 and PE11 (Table S4) and QuikChange II Site-Directed Mutagenesis Kit (200523, Agilent Technologies, Inc., Santa Clara, CA, USA), following the kit's instructions. The obtained entry clones with WT and K128R gene versions of ATG5 were then recombined into pCTAP vector (pYL436) under the control of 2x35S promoter [16] using Gateway cloning system (Invitrogen).

For generation of ATG5pr::ATG5-TAP constructs, the ATG5 gene AT5G17290.1 together with the promoter region was amplified using primers PE3 and PE4 (Table S4). The TAP tag was amplified from the pCTAP vector [16] using primers PE5 and PE9 (Table S4). The ATG5 gene and TAP tag were then fused using overlay PCR with primers PE3 and PE9 (Table S4). The obtained amplicon was cloned into pDONR/Zeo vector using Gateway cloning system (Invitrogen). The point mutation was introduced into this entry clone using primers PE10 and PE11 (Table S4) and QuikChange II Site-Directed Mutagenesis Kit (Agilent, 200523), according to the standard protocol. The obtained entry clones with WT and K128R gene versions of ATG5 genes including promoter region were then recombined into pGWB401 vector (Adgene, Plasmid #74795) using Gateway cloning system (Invitrogen).

Constructs for expressing fluorescent fusions of ATG5, ATG8, ATG16, ATG12A and ATG12B were produced using Gateway cloning system (Invitrogen). The corresponding genes or Coding DNA sequences were lifted from genomic DNA or total cDNA of *Arabidopsis* using primers provided in the Table S4 (PE8, SH139-145, AM 475, 476). Amplicons were recombined into pDONR/Zeo vecor to produce entry clones (see Table S4: SH 210-213, SH 231, 232 and AM 655). The entry clones were later recombined into pGWB-series destination vectors carrying 2x35S promoter and GFP, YFP or TagRFP tags (Adgene) (see Table S4, destination clones for expressing fluorescent fusions in plants).

The destination clones were used to transform *Agrobacterium tumefaciens* strain GV3101.

4.2. Protoplast and Plant Transformation

4.2.1. Transient Expression of *Arabidopsis* Protoplasts

Protoplasts were isolated from leaves of four-weeks-old plants expressing GFP-ATG8 in *atg5-1* using the "Tape-*Arabidopsis* Sandwich" method described in [71]. The isolated protoplasts were transformed using 10–20 μg of each plasmid (Table S4) using the method described in [72]. The transfected protoplasts were incubated in 24-well glass bottom plates (VWR CORN4441) for 16 h in light. The protoplasts were further treated with 5 μM AZD-8055 (364424, Santa-Cruz Biotech, Dallas TX, USA) and 0.5 μM Concanamycin A (202111A, Santa-Cruz Biotech) for 24 h, where applicable. The transformed protoplasts were imaged using CLSM800 (Carl Zeiss AG, Oberkochen BW, Germany), objective C-Apochromat

40×/1.2 W, excitation light 488 nm and 561nm and emission ranges of (515–560 nm) and (570–650 nm) for GFP and TagRFP, respectively. Images were analyzed using ZEN blue software (Carl Zeiss).

4.2.2. Transient Expression in Nicotiana Benthamiana

N. benthamiana plants were grown in 8 cm^3 pots filled with soil S-Jord (Hasselfors) under controlled growth conditions of 16 h light 8 h dark cycles, 70% relative humidity, light intensity of 150 µE m^{-2} s^{-1}, and day and night temperature of 22 °C and 20 °C, respectively. Transformed *A. tumefaciens* strain GV3101 (Rifampicin, 100 µg/mL) carrying the constructs of interest were grown in 5 mL of Luria-Broth high salt medium (L1704, Duchefa Biochemi, Haarlem, The Netherlands), supplemented with appropriate antibiotics (see Table S4, destination clones for expressing fluorescent fusions in plants). Liquid bacterial cultures were shaken at 200 rpm, 28 °C overnight, and then sedimented at 4000 G for five minutes. The resulting pellets were resuspended in MQ water with 150 µM Acetosyringone to the final OD_{600} = 0.15 and infiltrated in the abaxial side of leaves of five-week-old *N. benthamiana* plants. The leaves were imaged on the third day post-infiltration using CLSM800 (Carl Zeiss), objective C-Apochromat 40×/1.2 W, excitation light 488 nm and 561 nm and emission ranges of (515–560 nm) and (570–650 nm) for GFP and RFP, respectively. Images were analyzed using ZEN black software (Carl Zeiss).

4.2.3. Arabidopsis Thaliana Growth and Transformation

Arabidopsis plants were grown in 8 cm^3 pots filled with soil S-Jord (Hasselfors), under long day conditions: 150 µE m^{-2} s^{-1} light for 16 h, 8 h dark, 22 °C, 70% humidity.

Arabidopsis Col-0 wild-type plants and the previously described *atg5-1* mutant [73] were transformed using the standard "floral dip"-method [74]. *A. tumefaciens* strain GV3101 carrying the pYL436 and pGWB401 constructs (Table S4) was used for transformation. Transgenic plants were selected on Murashige and Skoog (MS) medium containing 40 µg mL^{-1} gentamycin and 50 µg mL^{-1} kanamycin, respectively. The plants were genotyped to confirm the presence of the transgenes using PE7 and PE8 primers (Table S4). Expression of the transgenes was confirmed using Western blot analyses (as described in the Section 4.3).

4.3. Immunoblotting

Plant material was powdered in liquid nitrogen, mixed with 2 vol. of hot 2× Laemmli buffer, and boiled for 10 min. Debris was pelleted for 5 min at 17,000 *G*. Proteins were separated on Mini-PROTEAN TGX stain-free Bio-Rad gels, 7.5% (Bio-Rad, Hercules, CA, USA) and transferred onto PVDF membranes. Membranes were blotted anti-actin 1:2000 (AS13 2640, Agrisera AB Vännäs, SWEDEN.), anti-myc 1:1000 (11667203001, Roche, Basel, Switzerland). Reactions were developed using Amersham ECL Prime kit (RPN2232, Cytiva, Marlborough, MA, USA) and detected using BioRad ChemiDoc.

4.4. Tandem Affinity Purification

Plants for affinity purification assay were grown in 8 cm^3 pots filled with soil S-Jord (Hasselfors) under long day conditions: 150 µE m^{-2} s^{-1} light for 16 h, 8 h dark, 22 °C, 70% humidity.

Plant rosette leaves were harvested at bolting stage and flash frozen in liquid nitrogen. Sample weight was >15 g of frozen tissue. Subsequent procedure was performed as previously described [18] using the following consumables: Protease inhibitor cocktail (P9599-5ML, Sigma-Aldrich, Saint Louis, MO, USA), PreScission protease (GE27-0843-01, Sigma-Aldrich), Ig-G sepharose beads (GE17-0969-01, Sigma-Aldrich),), Ni-sepharose beads (GE17-5318-01, Sigma-Aldrich).

Mass spectrometry data was acquired using a data-dependent acquisition procedure with a cyclic series of a full scan from 350–1500 with resolution of 120,000 control (AGC) target 1E6, maximum injection time 100 ms. The top S (3 s) and dynamic exclusion of 30 s

were used for selection of Parent ions for MSMS in the HCD cell with, relative collision energy 30% and scanned in the orbitrap with resolution of 30,000.

4.5. Mass Spectrometry

4.5.1. Liquid Chromatography and Mass Spectrometry Assay

LC-MS/MS was performed at the Mass Spectrometry Facility of Rutgers Center for Advanced Biotechnology and Medicine, US. Samples were loaded on to a fused silica trap column Acclaim PepMap100, 75 µm × 2 cm (Thermo Fisher Scientific, Waltham, MA USA).). After washing for 5 min at 5 µL/min with 0.1% TFA, the trap column was brought in-line with an analytical column (NanoeaseMZ peptideBEH C18, 130A, 1.7 µm, 75 mm × 250 mm, Waters, Milford, MA, USA) for LC-MS/MS. Peptides were fractionated at 300 nL/min using a segmented linear gradient 4–15% B in 30 min (where A: 0.2% formic acid, and B: 0.16% formic acid, 80% acetonitrile), 15–25% B in 40 min, 25–50% B in 44 min, and 50–90% B in 11 min. The column was re-equilibrated with 4% B for 5 min prior to the next run.

4.5.2. LC-MS/MS Data Analysis

Raw data files from the LC-MS/MS analysis were processed using MaxQuant (ver. 1.6.17.0) [19]. Default settings were used except enabling the protein label-free quantification (LFQ) and matching-between-runs options on default settings. Proteomics data was searched against the 26,755 representative Araport11 proteins [75] supplemented with the contaminant proteins list included within MaxQuant.

For the search including PTMs, phosphorylation (STY), acetylation of the side chain of Lys residues and protein N-terminal acetylation were specified in MaxQuant as variable modifications, while cysteine carbamidomethylation was set as fixed. Additionally, multiple events of methionine oxidation were detected, but not taken into consideration for this study, as it was not possible to discern whether they occurred in vivo or during samples processing [76].

4.5.3. Statistical Analysis of Interactors—MSqRobSum

The obtained MaxQuant peptide and protein result tables were used for statistical analysis in R (ver. 4.02) by MSqRobSum (ver. 0.0.0.9000) [77]. Proteins only identified by site and contaminant/decoy proteins were filtered. After default preprocessing, protein intensity summaries were estimated by robust regression, fitting $\log_2$ intensities in function of condition (WT ATG5, PM ATG5, and GFP control). Afterwards, user-defined contrasts of interest were tested to identify wild-type ATG5 (WT ATG5/GFP control) and mutated ATG (PM ATG5/GFP control) interactors. Interactors were filtered as having a fold change ≥ 1.5 and p value ≤ 0.05. In addition, proteins not quantified in GFP control conditions but identified in at least 2 out of 3 replicates of a ATG5 pulldown were manually curated as putative ATG5 interactors.

4.5.4. STRING Analysis

STRING analysis was made using https://string-db.org/ (accessed on 22 November 2022) website [78] with following parameters: full STRING network, active interaction sources: all possible; minimum required interaction score: medium confidence (0.400). Such settings were selected to include also possible connections that are not yet properly verified, which we found most suitable in the context of our search for unconventional roles of the ATG proteins. Suggested by STRING analysis connections were manually verified in the existing literature. When clustering was used it was with MCL clustering with an inflation parameter of 3.

5. Conclusions

This study provides novel evidence of PTMs occurring on core ATG proteins, i.e., ATG5 and ATG12 under non-inducing conditions. This discovery opens up exciting oppor-

tunities for further investigation and deeper understanding of the regulatory mechanisms governing ATG proteins in plants. Moreover, the comparative affinity purification assay revealed a compelling list of interactors for complete and minimal ATG5 complexes, indicating the possibility of crosstalk between autophagy-related proteins and multiple components of the endomembrane trafficking system and UPS. These findings strongly suggest the existence of shared and coordinated mechanisms that integrate these crucial cellular processes. Significantly, the identification of unique interactors for both ATG5 and ATG5^{K128R} suggests that ATG5 may possess roles and functions that extend beyond its classical involvement in autophagy. This finding underscores the complexity and versatility of ATG proteins and implies their potential contributions to diverse cellular pathways. To deepen our functional understanding of the complete and minimal ATG5 complexes, future studies should focus on verifying the identified interactors and conducting functional analyses of the candidate protein complexes. Additionally, we present novel evidence of nuclear localization for individual ATG5 and ATG12, that does not depend on their conjugation. Furthermore, we identified a set of nuclear-localized proteins, potential interactors of the nuclear fraction of ATG5 and ATG12. This discovery significantly expands the range of suggested functions associated with the components of the ATG5 complex. We hope that this study will pave the way for further explorations and place autophagic pathway into a broader context.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms241512300/s1, Table S1: MSqRob_without PTM; Table S2: MSqRob with PTM; Table S3: List of identified hits; Table S4: primers, plasmids and transgenic lines used in this study.

Author Contributions: Conceptualization, E.A.M. and P.V.B.; methodology, E.A.M., P.H.E. and P.W.; software, P.W.; validation, E.A.M., S.H. and P.H.E.; formal analysis, P.W., E.A.M., S.H. and P.H.E.; investigation S.H., P.H.E. and E.A.M.; resources, E.G.-B.; data curation, P.W., S.H. and E.A.M.; writing—original draft preparation, S.H., P.H.E. and E.A.M.; writing—review and editing, I.S., P.V.B. and P.W.; visualization, S.H., P.H.E., E.A.M. and I.S.; supervision, E.A.M. and P.V.B.; project administration, E.A.M. and P.V.B.; funding acquisition, E.A.M., P.V.B., P.W. and E.G.-B.; All authors have read and agreed to the published version of the manuscript.

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Autophagy, or "self-eating", is a highly conserved intracellular degradation and recycling process found in all eukaryotes. It plays a crucial role in maintaining cellular homeostasis by breaking down and recycling damaged or unwanted cellular components such as organelles and misfolded proteins. In this thesis, the plant-specific aspects of autophagy are explored with the aid of specialized in-house tools, elucidating the adaptation of the recycling process to autotrophic and heterotrophic organs.

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