Novel Insights into Vacuole-mediated Control of Plant Growth and Immunity

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2016

Acta Universitatis agriculturae Sueciae
2016:71

Cover: Confocal images showing co-localization of LAZ1-GFP (left, green) or LAZ1H1-GFP (right, green) with the tonoplast marker VAMP711-mCherry (magenta) in root epidermal cells.
(photo: Qinsong Liu)

ISSN 1652-6880
ISBN (print version) 978-91-576-8644-2
ISBN (electronic version) 978-91-576-8645-9

Print: SLU Service/Repro, Uppsala 2016

Nya Insikter i vakuol-medierad kontroll av växternas tillväxt och immunsvar

Sammanfattning

Växternas vakuoler är en form av organeller med viktiga funktioner rörande tillväxt, utveckling och stress-svar. De bidrar till att bibehålla cellers vätsketryck, till lagring av proteiner och andra substanser, och till utsöndring av kemiska försvarsämnen. Dessutom utgör vakuoler en del av cellens inre membransystem och har en roll i nedbrytningen av främmande ämnen som kommer in i cellen genom vesikulär transport och autofagi. Vakuolära och autofagiska processer antas också ha betydelse för hormonell signalering under tillväxt och immunsvar, liksom i regleringen av programmerad celldöd (PCD). Emellertid är de molekylära processer som ligger bakom en vakuol-medierad kontroll av tillväxt och immunsvar i stort sett okända, och denna avhandling har därför syftat till att öka förståelsen av dessa processer.

För detta ändamål karaktäriserades ett antal tidigare identifierade *lazarus* (*laz*)-suppressorer av konstitutiv celldöd i Arabidopsis-mutanten *accelerated cell death 11* (*acd11*). *LAZ4* kodar för retromer-komponenten VACUOLAR PROTEIN SORTING 35B (VPS35B). VPS35-proteiner visades bidra till vissa former av immunitetsrelaterad celldöd och sjukdomsresistens. En retromer-beroende vakuolär transport och integritet visades dessutom vara nödvändig för autofagi-processer under normala förhållanden, liksom under patogen-inducerad PCD.

En annan *LAZ*-suppressor, *LAZ1*, och dess närmaste homolog *LAZ1H1*, kodar för DUF300 domän-proteiner, och visades vara lokaliserade i det tonoplast-membran som omger vakuolen. Kombinerade mutationer av *LAZ1* och *LAZ1H1* ledde till en förändrad vakuolär morfologi, inhibering av tillväxt, och till en konstitutiv aktivering av hormonell brassinosteroid (BR) signalering. Vakuolär transport och nedbrytning av BR-receptorn BRI1 visades öka i *laz1 laz1h1*-mutanten, och var associerad med en ackumulering av BRI1s co-receptor BAK1. Eftersom andra typer av vakuolära mutanter visade normala BR-respons, föreslogs det att DUF300-proteiner i tonoplasten har en specifik roll i regleringen av BR-signalering genom att bibehålla den vakuolära integritet som krävs för att på subcellulär nivå balansera BAK1-nivåer och fördelning av BR-receptorn. Utöver ändrad vakuolär funktion och hormonell signalering, visades *laz1 laz1h1*-mutanten också ha en defekt basal autofagi. Eftersom den enkla *laz1*-mutanten visade en likande autofagisk defekt under bristförhållanden och immunitets-relaterad PCD, tyder resultaten på att LAZ1 är en central komponent för en fungerande autofagi.

Slutligen analyserades en inverkan av ökad autofagi på växtens produktivitet och stress-tolerans. Överuttryck i Arabidopsis av de autofagi-relaterade generna *ATG5* eller *ATG7* visades stimulera autofagi. Detta ledde i sin tur till en stimulerad immunitets-relaterad celldöd, och ökade toleransen mot oxidativ stress och nekrotrofiska svamp-patogener. Dessutom förbättrades även vegetativ tillväxt och fröproduktion. En genetisk stimulering av autofagi-processen kan därigenom komma att utnyttjas för att stärka ett brett spektrum av agronomiskt viktiga egenskaper, utan en samtidig minskning av reproduktiv kapacitet.

Novel Insights into Vacuole-mediated Control of Plant Growth and Immunity

Abstract

Plant vacuoles are organelles with numerous biological functions in growth, development, and stress responses. These include maintenance of turgor pressure, storage of minerals and proteins, and degradation of cellular content delivered by endosomal trafficking and autophagy pathways. Intriguingly, vacuolar and autophagic processes have been implicated in hormone signaling during growth and immune responses, and in the regulation of programmed cell death (PCD). However, the molecular players and mechanisms underlying the vacuole-mediated control of growth and immunity remain poorly understood, and this thesis therefore aimed at improving our understanding of these systems.

For this purpose, previously isolated *lazarus* (*laz*) suppressors of constitutive cell death in the Arabidopsis mutant *accelerated cell death 11* (*acd11*) were characterized. *LAZ4* encodes the retromer component VACUOLAR PROTEIN SORTING 35B (VPS35B). VPS35 proteins were found to contribute to certain forms of immunity-related cell death and disease resistance. Furthermore, retromer-dependent vacuole trafficking and integrity were shown to be essential for autophagy processes under basal and immunity-associated conditions.

Another *LAZ* suppressor, *LAZ1*, and its closest homolog *LAZ1H1* encode DUF300 domain-containing proteins and were found to localize to the tonoplast. Combined loss-of-function mutations in *LAZ1* and *LAZ1H1* resulted in altered vacuole morphology, growth inhibition, and constitutive activation of brassinosteroid (BR) hormone signaling. Vacuolar trafficking and degradation of the BR receptor BRI1 were shown to be enhanced in the *laz1 laz1h1* mutant and associated with increased tonoplast accumulation of the BRI1 co-receptor BAK1. Since unrelated vacuole mutants exhibited normal BR responses, tonoplast DUF300 proteins were suggested to play distinct roles in the regulation of BR signaling. In addition, *laz1 laz1h1* plants were impaired in basal autophagy. Since the *laz1* single mutant showed a similar autophagic defect upon starvation and immunity-related PCD, LAZ1 was proposed to be the main contributor to autophagy function.

Finally, the impact of enhanced autophagy on plant productivity and stress tolerance was analyzed. Constitutive overexpression of the autophagy-related genes *ATG5* or *ATG7* in Arabidopsis was shown to stimulate autophagy flux, which promoted immunity-related cell death and enhanced resistance to oxidative stress and necrotrophic fungal pathogens. Furthermore, increased autophagy improved vegetative growth and increased seed production. Therefore, genetic enhancement of autophagy levels could be potentially used in plants to improve various agronomically important traits.

Keywords: Arabidopsis, vacuole, retromer, DUF300 proteins, autophagy

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Dedication

To my parents.

Keep what you say and carry out what you do.
Confucius

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 Munch, D.*, Teh, O.K.*, Malinovsky, F.G.*, **Liu Q.**, Vetukuri, R.R., El Kasmi, F., Brodersen, P., Hara-Nishimura, I., Dangl, J.L., Petersen, M., Mundy, J. & Hofius D. (2015). Retromer contributes to immunity-associated cell death in Arabidopsis. *The Plant Cell* 27, 463-479. * These authors contributed equally to the work.

II **Liu, Q.**, Vain, T., Viotti, C., Tarkowská, D., Novák, O., Sitbon, F., Robert, S. & Hofius D. (2016). Arabidopsis DUF300 proteins at the tonoplast are required for regulation of brassinosteroid hormone signaling. (Submitted).

III **Liu, Q.**, Hafrén, A., Andersen, S.U. & Hofius D. The tonoplast DUF300 protein LAZ1 is required for immunity-asscociated autophagy in Arabidopsis. (Manuscript).

IV Minina, E.A., Moschou, P.N.*, Vetukuri, R.R.*, Sanchez-Vera, V.*, **Liu, Q.***, Beganovic, M., Yilmaz, J.L., Shabala, L., Suarez, M.F., Shabala, S., Stymne, S., Hofius, D. & Bozhkov P.V. (2016) Transcriptional stimulation of autophagy improves plant fitness. (Submitted). * These authors contributed equally to the work.

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Additional publications

Mozgová, I.*, Wildhaber, T.*, **Liu, Q.**, Abou-Mansour, E., L'Haridon, F., Métraux, J.P., Gruissem, W., Hofius, D. & Hennig, L. (2015) Chromatin assembly factor CAF-1 represses priming of plant defence response genes. *Nature Plants* 1:15127. * These authors contributed equally to the work.

The contribution of Qinsong Liu to the papers included in this thesis was as follows:

I Established the "autophagy flux" assay based on NBR1 immunoblotting, which was subsequently used to analyse autophagy defects in retromer-deficient mutants. Performed resistance assays with virulent and avirulent bacterial strains.

II Participated in planning of the study, performed all laboratory work except for TEM, and wrote the first draft of the manuscript.

III Participated in planning of the study, performed all laboratory work except for hydroxyurea resistance assay and ELISA, and wrote the first draft of the manuscript.

IV Contributed to the analysis of disease resistance and cell death in plant lines with enhanced autophagy.

1 Introduction

Plant vacuoles are multifunctional organelles that play important roles in plant development and growth. Vacuoles occupy most of the plant cell volume (up to 90%) and control turgor pressure required for cell expansion (Zhang *et al.*, 2014; Marty, 1999). In addition, they are involved in the storage of a large variety of substances (e.g. minerals, nutrients, proteins, and secondary metabolites), which allow plant cells to maintain pH homeostasis, balance fluctuations in nutrient availability, sequester harmful compounds, and respond to various stress conditions including pathogen infection (Olbrich *et al.*, 2007; Paris *et al.*, 1996). Vacuoles also function as lytic compartment to degrade cellular cargoes derived from two major intracellular trafficking pathways, endocytosis and autophagy (Zhuang *et al.*, 2015). Based on these properties, vacuoles are increasingly recognized for their roles in cellular signaling during growth and immune responses and in the regulation of programmed cell death (PCD) (Baster *et al.*, 2013; Beck *et al.*, 2012; Hara-Nishimura & Hatsugai, 2011; Kasai *et al.*, 2011; Nimchuk *et al.*, 2011; Hatsugai *et al.*, 2009; Hatsugai *et al.*, 2004).

During recent years, tremendous progress has been made in unraveling vacuole-related functions, components, and pathways by proteomic and metabolomic analyses of vacuole content as well as biochemical and genetic characterization of vacuole-associated proteins (Jiskrova *et al.*, 2016; Zhang *et al.*, 2014; Ranocha *et al.*, 2013; Trentmann & Haferkamp, 2013; Martinoia *et al.*, 2012; Schmidt *et al.*, 2007). However, many of the signals and molecular pathways that govern vacuole-mediated control of the multiple biological processes remain elusive, thus encouraging further investigation.

This thesis work aimed to study how vacuole-associated proteins and trafficking pathways (e.g. autophagy) regulate two important aspects of plant life, growth and immunity. The following parts are therefore intended to introduce a rather broad spectrum of research areas that are relevant for the

different subprojects of the thesis, including endomembrane trafficking, autophagy, innate immunity, cell death, and brassinosteroid signaling.

1.1 Plant endomembrane system and membrane trafficking pathways

Eukaryotes possess cellular membranes that are functionally inter-related and inter-connected, thereby forming the endomembrane system. The endomembrane system is crucial for the exchange and transport of materials such as proteins and lipids within cells, and generally includes the plasma membrane, the Golgi apparatus, the endoplasmic reticulum (ER), the nuclear envelope, endosomes, and lytic compartments. The endomembrane system and membrane trafficking in plant cells share a number of important characteristics with other eukaryotic organisms, but also exhibit some complex and unique features (Cheung & de Vries, 2008; Jurgens, 2004). In general, plant membrane trafficking pathways include endocytosis (internalization from the plasma membrane/extracellular space to other subcellular compartments), exocytosis (delivering cargoes to the plasma membrane/extracellular milieu), and vacuolar transport (Figure 1). These trafficking routes converge at a common sorting hub [known as *trans*-Golgi network (TGN)/early endosome (EE)] from where the cargoes destined for degradation and recycling are separated (Robinson *et al.*, 2008). Another well-characterized endosomal compartment in plant cells is the multivesicular body (MVB)/prevacuolar compartment (PVC)/late endosome (LE), which is known to originate from the TGN/EE and mediate the transport of vacuolar cargo via MVB-vacuole fusion (Singh *et al.*, 2014; Scheuring *et al.*, 2011).

1.1.1 Endocytosis

Uptake experiments with fluorescent and membrane-impermeant molecules allowed direct visualization and quantification of endocytosis. The amphiphilic styryl FM (Fei Mao) dyes developed by Betz and co-workers have been routinely used as endocytosis markers in eukaryotic cells (Jelinkova *et al.*, 2010; Betz *et al.*, 1996; Betz *et al.*, 1992). Mounting evidence suggests that live imaging of FM4-64 is an efficient and reliable method to study organelle organization and particularly endocytic pathways in plants. FM4-64 initially stains the plasma membrane and follows internalization processes primarily by endocytic vesicles. Subsequently, FM4-64 is distributed throughout the whole vesicle trafficking network and finally ends up on the membrane surrounding the vacuole, the tonoplast (Figure 1) (Rigal *et al.*, 2015; Dettmer *et al.*, 2006; Kutsuna & Hasezawa, 2002).

To date, the best-studied endocytic pathway in eukaryotic organisms utilizes the vesicle coat scaffold protein clathrin, and is therefore designated as clathrin-mediated endocytosis (CME). The functions of a number of components involved in CME have been extensively investigated in mammals (McMahon & Boucrot, 2011; Traub, 2009). In contrast, knowledge about the CME machinery in plants is still in its infancy. Plants seem to possess all of the molecular components that are required for a functional CME pathway. For instance, the Arabidopsis genome encodes multiple CLATHRIN HEAVY CHAIN (CHC) and CLATHRIN LIGHT CHAIN (CLC) proteins, all subunits of the heterotetrameric ADAPTOR PROTEIN COMPLEX-2 (AP-2) which act as the major adaptor for CME, as well as accessory proteins (Chen *et al.*, 2011). Notably, both genetic and pharmacological approaches in plants have revealed important roles of CME in the regulation of hormonal signaling, nutrient homeostasis, and defence responses. Accordingly, CME has been shown to constitute the predominant internalization process for assorted endocytic cargoes, including the BRASSINOSTEROID INSENSITIVE1 (BRI1)-ligand complex, PIN-FORMED (PIN) auxin transporters, the boron transporter BOR1, the iron transporter IRT1, and the immunity-associated receptor ETHYLENE-INDUCING XYLANASE 2 (LeEIX2) (Gadeyne *et al.*, 2014; Adam *et al.*, 2012; Irani *et al.*, 2012; Barberon *et al.*, 2011; Sharfman *et al.*, 2011; Takano *et al.*, 2010; Dhonukshe *et al.*, 2007).

1.1.2 Exocytosis

Constitutive cycling of proteins between the cell surface and TGN compartment relies on the coordinated action of endocytosis and exocytosis, and regulates their abundance and polar localization in response to internal and external cues (Zarsky *et al.*, 2009). In contrast to endocytosis, however, the regulation of plant exocytosis is much less understood.

The fungal toxin brefeldin A (BFA) which inhibits the functions of vesicle budding regulators ARF-GEFs (ADP ribosylation factor guanine nucleotide exchange factors) has been commonly used to study endomembrane trafficking (Geldner *et al.*, 2003; Geldner *et al.*, 2001). Importantly, the best-characterized ARF-GEF is GNOM, which was shown to be sensitive to BFA and mediate endocytosis and exocytosis of PIN proteins (Naramoto *et al.*, 2010; Geldner *et al.*, 2003). Depending on the concentration used, 25 μM BFA can specifically disrupt exocytosis/recycling processes to the plasma membrane (Figure 1), while 50 μM also interferes with vacuolar transport (Robert *et al.*, 2010; Kleine-Vehn *et al.*, 2008). Consequently, endocytosed materials accumulate in intracellular agglomerations, termed BFA compartments, mainly comprising early secretory compartments (Grebe *et al.*, 2003; Geldner *et al.*, 2001). After

wash-out of BFA, these materials are recycled from BFA compartments to the plasma membrane via an exocytotic event (Geldner *et al.*, 2001). Therefore, BFA treatment is considered as an effective tool to identify regulators involved in endocytosis and/or exocytosis.

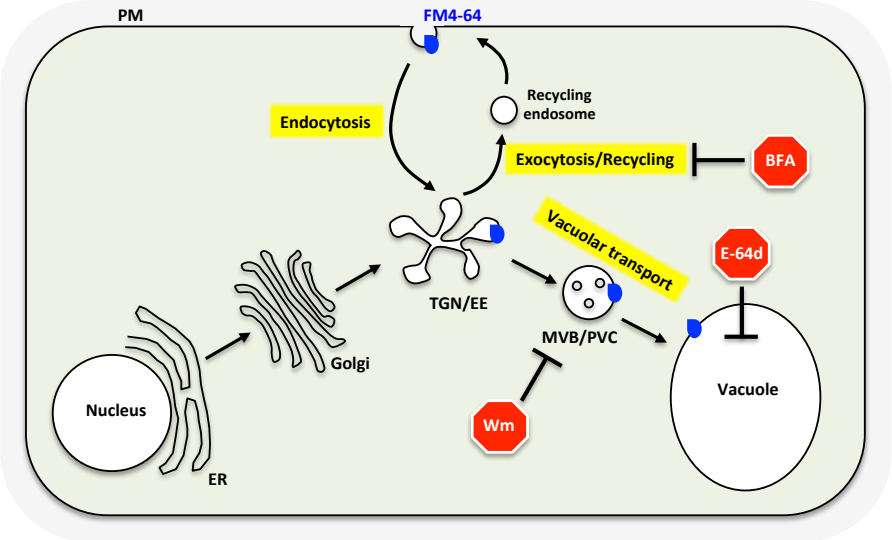

Figure 1. Schematic diagram presenting the plant endomembrane system as well as the trafficking pathways including endocytosis, exocytosis/recycling, and vacuolar transport. The endomembrane system in plants generally includes the plasma membrane (PM), the nucleus, the endoplasmic reticulum (ER), the Golgi apparatus, the *trans*-Golgi-network/early endosome (TGN/EE), the multivesicular body/prevacuolar compartment (MVB/PVC), and the vacuole. A variety of inhibitors including brefeldin A (BFA), wortmannin (Wm), and E-64d have been commonly used to study endomembrane trafficking in plants: (i) BFA can specifically disrupt exocytosis/recycling processes to the PM (Geldner *et al.*, 2003; Geldner *et al.*, 2001), (ii) Wm can cause enlargement of the MVB/PVC through inactivation of phosphatidylinositol 3-kinases (Matsuoka *et al.*, 1995), and (iii) E-64d inhibits the activity of the cysteine proteases and thus blocks the vacuolar degradation of proteins (Bassham, 2015). The lipophilic styryl dye FM4-64 has been widely exploited to investigate organelle organization and particularly endocytic pathways in plants (Rigal *et al.*, 2015). FM4-64 initially stains the PM, follows internalization processes, and finally ends up on the tonoplast.

Apart from BFA, other inhibitors have been reported to affect exocytosis. As demonstrated by BFA wash-out experiments, the compound Endosidin5 (ES5) suppresses exocytosis of PIN proteins, which leads to enhanced trafficking to the vacuole (Drakakaki *et al.*, 2011). Recently, another small chemical inhibitor, Endosidin2 (ES2), was shown to target a member of the EXO70 (exocyst component of 70 kDa) family to block exocytosis in both plants and mammals (Zhang *et al.*, 2016a). EXO70 is a subunit of the octameric exocyst complex, which controls the last exocytosis steps (i.e.

tethering of secretory vesicles to the plasma membrane) in eukaryotic cells (Synek *et al.*, 2014; Heider & Munson, 2012). Cross-kingdom characterization of ES2 as a specific exocytosis inhibitor and identification of its target will largely facilitate our understanding of exocytosis regulation, and may also help to develop new drugs for exocyst-related diseases.

1.1.3 Vacuolar trafficking and degradation

The fate of endocytosed plasma membrane cargoes can substantially differ as they are either recycled back from TGN to the plasma membrane or targeted via MVB/PVC to the vacuole for turnover. In general, vacuolar trafficking and degradation of proteins seem to be tightly regulated by developmental and growth-related cues or environmental changes. For instance, gravity stimulation, darkness as well as cytokinin and auxin hormones were shown to promote vacuolar transport and destruction of PIN proteins (Baster *et al.*, 2013; Marhavy *et al.*, 2011; Kleine-Vehn *et al.*, 2008; Laxmi *et al.*, 2008). In addition, BOR1 and IRT1 transporters undergo ubiquitination-dependent trafficking to the vacuole when exposed to their respective minerals (Barberon *et al.*, 2011; Kasai *et al.*, 2011; Takano *et al.*, 2005). Similarly, recent data indicate that endocytosis and vacuolar transport of the BRI1 receptor are controlled by ubiquitination at the cell surface, but occur independently of ligand binding (Martins *et al.*, 2015; Geldner *et al.*, 2007).

Prior to delivery to vacuoles, recognition of ubiquitinated cargo proteins and their sorting into the luminal vesicles of MVB/PVC/LE is mediated by ENDOSOMAL COMPLEX REQUIRED FOR TRANSPORT (ESCRT) system, which usually consists of four evolutionary conserved subunits (i.e. ESCRT-0, -I, -II, and -III) (Raiborg & Stenmark, 2009). Besides ESCRT, retromer is another important component in the regulation of vacuolar trafficking (Nodzynski *et al.*, 2013; Kleine-Vehn *et al.*, 2008). It is known that retromer in mammals is constituted by two functionally distinct subcomplexes: the trimeric core retromer [including VACUOLAR PROTEIN SORTING 29 (VPS29), VPS35, VPS26] and a dimer of sorting nexins (SNXs) (Attar & Cullen, 2010). Notably, the Arabidopsis genome harbors homologous genes encoding all the retromer components (Robinson *et al.*, 2012). In plants, retromer subunits were shown to localize to MVB/PVC and function in recycling of vacuolar sorting receptors (VSRs) (Kang *et al.*, 2012; Yamazaki *et al.*, 2008; Jaillais *et al.*, 2007; Oliviusson *et al.*, 2006). Due to retromer-mediated retrieval, these receptors are able to escape lytic degradation and act in the next round of sorting. Furthermore, retromer components are involved in maintaining endosome homeostasis, PIN protein recycling, and protein sorting to protein storage vacuoles (PSVs) (Yamazaki *et al.*, 2008; Jaillais *et al.*,

2007). These functions integrate the retromer trafficking machinery into plant cell polarity, organ emergence, and seed storage. In addition, retromer was recently shown to be crucial for oil body (OB) formation, lipid storage and breakdown, and correct movement of the major lipase SUGAR-DEPENDENT1 (SDP1) to the OB surface (Thazar-Poulot *et al.*, 2015). Although extensive work established functions of plant retromer in the developmental context, other potential roles with regard to disease resistance and pathogen-triggered cell death still need to be dissected.

1.1.4 Tonoplast proteins and functions

In plant cells, the vacuole is surrounded by a membrane barrier known as the tonoplast, which separates vacuole lumen from cytoplasm and mediates the exchange between them. Tonoplast-resident proteins are key regulators of vacuolar functions and are required for membrane fusion events and transport processes (Zhang *et al.*, 2014; Martinoia *et al.*, 2012).

Rab7-like proteins are regulators with conserved functions in membrane trafficking in mammalian systems (Zhang *et al.*, 2009). The Arabidopsis genome contains eight genes encoding Rab7-like proteins including RabG3c and RabG3f (Vernoud *et al.*, 2003). Due to the potential redundancy and functional compensation within the Rab7 family, dominant-negative rather than loss-of-functions mutations have been exploited to investigate the role of individual members. It was demonstrated that RabG3c localizes to the tonoplast and that its dominant-negative form blocks the terminal delivery to the lytic vacuole (Bottanelli *et al.*, 2011). In contrast, RabG3f showed dual localization to MVB/PVC and the tonoplast and its dominant-negative form caused enlarged MVB/PVC and fragmented vacuoles, impaired vacuolar targeting, and a seedling-lethal phenotype (Cui *et al.*, 2014). In addition, the MONENSIN SENSITIVITY1 (MON1)/SAND- CALCIUM CAFFEINE ZINC SENSITIVITY1 (CCZ1) complex, which acts as an effecter of Rab5, was shown to be responsible for activation of RabG3f (Cui et al., 2014; Singh et al., 2014) (Cui *et al.*, 2014; Singh *et al.*, 2014). Interestingly, RabG3f can directly interact with the retromer component VPS35A, implying the possibility that recruitment of the plant core retromer complex from cytosol to the endosomal membrane is facilitated by this interaction (Zelazny *et al.*, 2013).

Apart from Rab7-like proteins, the functions of other types of tonoplast-localized proteins have been reported. SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins are vital for vesicle fusion and exist in plants as a large family (Sanderfoot *et al.*, 2000). The Qa-SNARE protein, VAM3/SYP22, was found to reside predominantly on the tonoplast and MVB/PVC (Sanderfoot *et al.*, 1999; Sato *et al.*, 1997). Expression of

Arabidopsis VAM3/SYP22 rescued the vacuole morphology defects of the yeast *vam3* mutant, highlighting the conservation of VAM3/SYP22 function (Sato *et al.*, 1997). Moreover, VAM3/SYP22 and its close homologue PEP12/SYP21 possess redundant and compensatory functions during plant development (Uemura *et al.*, 2010). Another SNARE protein, VAMP711, was originally identified by a proteomics approach as part of the SNARE complex that mediates fusion of vesicles with the tonoplast (Carter *et al.*, 2004). Due to its distinct association with the vacuole membrane, fluorescent protein-tagged VAMP711 has been widely used as a tonoplast marker (Geldner *et al.*, 2009). A recent study characterized a group of tonoplast-associated phosphatases, named SUPPRESSOR OF ACTIN (SAC) proteins, which participate in polyphosphoinositide (PPI) metabolism, maintain normal vacuole morphology, and regulate vacuolar targeting (Novakova *et al.*, 2014). Importantly, treatment with PPI leads to smaller sized fragmented vacuoles, strongly resembling the vacuolar morphology in untreated *sac* loss-of-function mutants (Novakova *et al.*, 2014). This work revealed that PPIs and their metabolic enzymes SACs are of critical importance for vacuolar functions. In addition, Arabidopsis VACUOLELESS1 (VCL1), the homologue of yeast Vps16p, localizes to the tonoplast and MVB/PVC (Rojo *et al.*, 2003). Remarkably, loss of VCL1 was reported to cause the absence of vacuoles, induce the accumulation of autophagosomes, and result in embryonic lethality (Rojo *et al.*, 2001).

It has been suggested that various important biological processes are dependent on a large number of tonoplast-localized transporters (Martinoia *et al.*, 2012). These processes include the exchange of nutrients and minerals in response to nutrient deficiencies and environmental changes, the accumulation of secondary metabolites and defence compounds, as well as the sequestration of toxic compounds. Increasing evidence further suggests that vacuolar transporters contribute to hormone homeostasis in plants. For instance, abscisic acid glucosyl ester (ABA-GE), the major glucose conjugate of abscisic acid (ABA), was found to accumulate exclusively in vacuoles of plant cells (Piotrowska & Bajguz, 2011; Lehmann & Glund, 1986). Intriguingly, the import of the ABA conjugate into the vacuole is mediated by transport mechanisms that engage ATP-BINDING CASSETTE (ABC) transporters and the proton gradient (Burla *et al.*, 2013). In addition, indole-3-acetic acid (IAA) and other related compounds have recently been identified in purified Arabidopsis vacuoles, and the plant unique protein WALLS ARE THIN 1 (WAT1) was shown to mediate auxin export from vacuoles (Ranocha *et al.*, 2013). These findings indicated that the vacuole is indispensable for the regulation of intracellular auxin levels and homeostasis (Ranocha *et al.*, 2013).

In plant cells, active transport of solutes across the vacuolar membrane requires combined action of two types of proton pumps, the vacuolar H^+-ATPase (V-ATPase) and the vacuolar H^+-pyrophosphatase (V-PPase). In addition to their role in energizing transport processes, V-ATPase and the V-PPase have been implicated in pH-dependent endomembrane trafficking (Schumacher, 2014). In Arabidopsis, the distinct subcellular distribution of V-ATPase is conferred by three isoforms of the membrane-integral subunit VHA-a: the TGN/EE-associated VHA-a1 and tonoplast-localized VHA-a2 and VHA-a3 (Krebs *et al.*, 2010; Dettmer *et al.*, 2006). Combined loss-of-function mutations in *VHA-a2* and *VHA-a3* lead to the lack of tonoplast V-ATPase activity, elevated vacuolar pH, a daylength-dependent dwarf phenotype, and compromised capacity for nutrient storage (Krebs *et al.*, 2010). Furthermore, V-ATPase activity in the TGN/EE, but not at the tonoplast, is important for salt tolerance and exocytosis/recycling (Luo *et al.*, 2015; Krebs *et al.*, 2010). Recently, it has been shown that constitutive up-regulation of V-PPase is unable to compensate for the loss of tonoplast V-ATPase function, but augmented V-ATPase activity triggered by cold acclimation is V-PPase-dependent (Kriegel *et al.*, 2015). Intriguingly, the mutant deficient in both tonoplast V-ATPase and V-PPase is viable and maintains vacuole acidification, thus providing a valuable genetic tool to study how TGN/EE-associated V-ATPase contributes to vacuolar pH (Kriegel *et al.*, 2015).

Despite the described advances in the identification and characterization of tonoplast-resident transporters, enzymes, and fusion-related proteins, the nature and functions of many tonoplast proteins remain to be determined (Trentmann & Haferkamp, 2013; Martinoia *et al.*, 2012; Schmidt *et al.*, 2007).

1.2 Autophagy

Autophagy ("self-eating") is a major intracellular trafficking and degradation system conserved among eukaryotes. Autophagic mechanisms mediate either the bulk degradation of intracellular content or selective clearance of damaged organelles, protein aggregates, and lipids (Yang & Klionsky, 2010). At basal levels, autophagy contributes to housekeeping function in cellular homeostasis, whereas augmented autophagy activity facilitates adaptation and cell survival in response to stress conditions (Reggiori & Klionsky, 2013). Initially discovered in yeast, autophagy was subsequently shown to play paramount roles in a wide range of processes in animals, including development, starvation adaptation, tissue homeostasis, senescence and aging, programmed cell death, immunity and disease (Nixon, 2013; Mizushima *et al.*, 2011; Di Bartolomeo *et al.*, 2010). In plants, the mechanisms underlying autophagy have

also been extensively studied during the past few years, which highlighted the importance of autophagy in many aspects of plant life (Michaeli *et al.*, 2016; Liu & Bassham, 2012).

1.2.1 Autophagy machinery

The principal feature of autophagy is the formation of double membrane-bound vesicles, termed autophagosome, that entraps and delivers cytosolic cargoes to the vacuole/lysosome for degradation and recycling (Mizushima, 2007). Autophagosome initiation and completion are carried out by a repertoire of AUTOPHAGY-RELATED (ATG) proteins (Levine & Klionsky, 2004). *ATG* genes were first discovered by genetic screens in yeast, and more than 35 *ATG* genes have been functionally characterized (Shibutani & Yoshimori, 2014). Many of these genes have close homologues in other organisms and the conserved core set of ATG proteins can be separated into functional units that regulate distinct steps of the autophagy pathway (Shibutani & Yoshimori, 2014; Mizushima, 2007; Xie & Klionsky, 2007). The ATG1-ATG13 kinase complex is responsible for autophagy induction and is negatively regulated by the target of rapamycin (TOR) kinase. The class III phosphatidylinositol 3-kinase (PI3K) complex harbors Beclin1/ATG6 and plays a crucial role in vesicle nucleation. The ATG9-ATG2-ATG18 transmembrane complex is generally considered to recycle and retrieve autophagy proteins, and to provide membranes from various sources (e.g. mitochondria, ER, and TGN) to the expanding phagophore. In addition, two ubiquitin-like (UBL) conjugation pathways contribute to autophagosome biogenesis by producing ATG12-ATG5 and ATG8-phosphatidylethanolamine (PE) conjugates. The ATG8 conjugation pathway requires the cysteine proteinase ATG4 (belonging to the caspase family), as well as the E1-like activating enzyme ATG7 (for further details, see Figure 2).

1.2.2 Markers for autophagosomes in plant cells

It is well established that the PE-conjugated ATG8 is tightly associated with the autophagosome from its initiation to lytic degradation (Figure 2) (Xie & Klionsky, 2007). Hence, the subcellular distribution of fluorescent protein-tagged ATG8 fusions, together with the conversion of soluble to membrane-bound ATG8, has been commonly exploited to monitor autophagosome formation and autophagy activity in plants (Bassham, 2015).

In plants, the mechanisms of autophagosome formation at early stages are not well characterized and understood. Elegant imaging studies using fluorescent protein-tagged ATG5 were recently explored to identify growing phagophores, also known as the isolation membrane (Le Bars *et al.*, 2014). In

this work, ATG5 was shown to locate to the outer surface of the cortical endoplasmic reticulum (ER) and to rapidly anchor ATG8 to the initial phagophore, which is generally believed to be essential for membrane expansion (Xie *et al.*, 2008; Nakatogawa *et al.*, 2007). Furthermore, it was demonstrated that ATG5 continuously decorates the edges of the expanding phagophore and develops into a torus-like structure on the aperture of the cup-shaped membrane structure. As soon as the phagophore aperture is sealed, ATG5 leaves the structure and the newly formed autophagosome is simultaneously dissociated from the ER (Le Bars *et al.*, 2014).

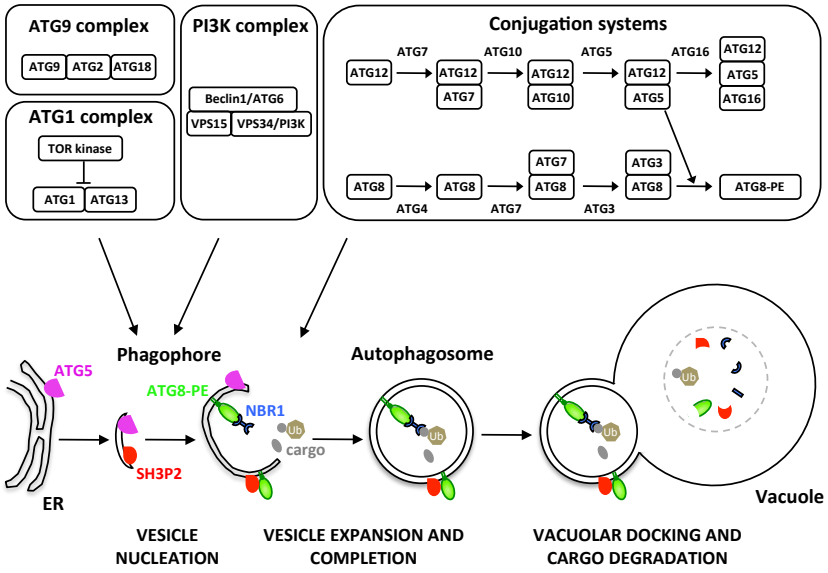

Figure 2. Schematic overview of the autophagy pathway in plants. Autophagy is initiated by nucleation and expansion of the initial sequestering compartment, the phagophore. Subsequently, the outer membrane of the autophagosome fuses with the tonoplast, which results in release of the inner single-membrane vesicle, referred to as the autophagic body, into the vacuolar lumen for its breakdown. Lipidation of ATG8 and vacuolar degradation of the autophagic adaptor protein NBR1 have been routinely used for monitoring autophagosome formation and autophagic flux. In plants, it is well documented that SH3P2 and ATG5 are important regulators of autophagosome formation. The regulation of autophagy induction requires the action of the TOR kinase and ATG1 complex. The class III PI3K complex harboring Beclin1/ATG6 plays a crucial role in vesicle nucleation. In addition, The ATG9 complex is generally considered to recycle and retrieve autophagy proteins, and to provide membranes from various sources to the expanding phagophore. The ATG12-ATG5-ATG16 complex and ATG8-PE adduct produced by two ubiquitin-like conjugation pathways are essential for membrane elongation and autophagosome formation. Modified from (Hofius *et al.*, 2011).

A non-ATG protein with a Bin-Amphiphysin-Rvs (BAR) domain, SH3 DOMAIN-CONTAINING PROTEIN2 (SH3P2), was additionally reported to

label phagophores at the early stage and modulate autophagosome biogenesis (Zhuang *et al.*, 2013). Under autophagy-inducing conditions, SH3P2 is localized to the phagophore assembly site (PAS, also known as pre-autophagosomal structure). However, in contrast to ATG5, SH3P2 signals can be also observed in the vacuolar lumen, indicating that it remains associated with autophagosomal structures during vacuolar turnover. Moreover, SH3P2 appears to promote autophagosome formation through association with phosphatidylinositol 3-phosphate (PI3P), PI3K complex, and ATG8.

Therefore, the combined analysis of fluorescent protein-tagged ATG8, SH3P2, and ATG5 proteins is emerging as effective and reliable tool to monitor autophagosome dynamics, ranging from its formation to vacuolar breakdown (Figure 2) (Bassham, 2015).

1.2.3 Selective autophagy in plants

The best-characterized form of selective autophagy in the plant system is NEIGHBOR OF BRCA1 GENE1 (NBR1)-mediated degradation of protein aggregates (Zhou *et al.*, 2013; Svenning *et al.*, 2011). It has been demonstrated that plant NBR1 represents the functional hybrid of mammalian p62 and NBR1 proteins based on its ability to homopolymerize (Svenning *et al.*, 2011). NBR1 acts as autophagy cargo receptor and plays a pivotal role in the disposal of ubiquitinated proteins accumulated during different stress conditions (Figure 2) (Zhou *et al.*, 2013; Svenning *et al.*, 2011). NBR1 possesses a conserved LC3-interacting region [LIR, also known as ATG8-interacting motif (AIM)] that binds to membrane-bound lipidated ATG8, as well as a C-terminal ubiquitin-associated (UBA) domain capable of targeting ubiquitinated protein aggregates for autophagic degradation (Zhou *et al.*, 2013; Svenning *et al.*, 2011). Importantly, NBR1 itself is an autophagic substrate and accumulates in autophagy deficient mutants (Svenning *et al.*, 2011). Therefore, vacuolar degradation of NBR1 has been frequently used to monitor autophagic flux under certain biotic and abiotic conditions (Coll *et al.*, 2014; Hackenberg *et al.*, 2013; Minina *et al.*, 2013).

Another form of selective autophagy engages the plant unique protein ATG8-INTERACTING PROTEIN 1 (ATI1), which was initially identified as ATG8f-interacting protein (Honig *et al.*, 2012). Interestingly, ATI1 is translocated during senescence to plastid-associated bodies (known as ATI1-PS bodies), where it binds both plastid proteins and ATG8 to mediate their vacuolar turnover (Michaeli *et al.*, 2014). Hence, ATI1 seems to function as cargo receptor in a plastid-to-vacuole membrane trafficking route that relies on autophagy mechanisms.

TRYPTOPHAN-RICH SENSORY PROTEIN (TSPO) was proposed to function as an additional cargo receptor of selective autophagy processes in Arabidopsis (Hachez *et al.*, 2014; Vanhee *et al.*, 2011). TPSO contains an AIM domain and was previously shown to be degraded by autophagy under stress-induced conditions (Vanhee *et al.*, 2011). Notably, TPSO interacts with the aquaporin PLASMA MEMBRANE INTRINSIC PROTEIN 2;7 (PIP2;7), which normally cycles between the plasma membrane and endosomes (Hachez *et al.*, 2014). However, in response to abiotic stress, PIP2;7 is degraded by autophagy in a TPSO-dependent manner, thereby modulating PIP2;7-mediate water transport processes.

Recently, the selective autophagic degradation of defective 26S proteasomes, termed proteaphagy, in response to MG132 inhibitor treatment and starvation has been reported (Marshall *et al.*, 2015). The proteasome subunit REGULATORY PARTICLE NON-ATPASE 10 (RPN10) was identified as cargo receptor, which binds both lipidated ATG8 and ubiquitinated proteasomes. These findings are fundamental to advance our understanding of the cross-talk between the two major cellular degradation systems.

1.2.4 Interplay between autophagic and late endocytic membrane trafficking

Autophagic and endocytic cargoes destined for vacuolar degradation are delivered into the vacuolar lumen via membrane fusion. In non-plant systems, such as animals and yeast, autophagosomes have been reported to fuse either directly with lysosomes/vacuoles or with MVB/PVC to form intermediate organelles known as amphisomes, which later merge with the lytic compartments for subsequent degradation. Notably, a similar fusion event between autophagosomes and vacuoles has also been demonstrated to occur in plants (Zhuang *et al.*, 2015). There is accumulating evidence that dysfunction of certain regulators in the conventional MVB/PVC-vacuole trafficking route also affects autophagy in plants (Zhuang *et al.*, 2015).

The ESCRT system is responsible for sorting ubiquitinated cargo proteins into intraluminal vesicles (ILVs) of MVB/PVC (Raiborg & Stenmark, 2009). In Arabidopsis, several studies revealed that ESCRT functions are required for autophagosomal degradation (Gao *et al.*, 2015; Kolb *et al.*, 2015; Spitzer *et al.*, 2015; Katsiarimpa *et al.*, 2013). For instance, overexpression of a dominant-negative form of the ESCRT-III subunit VACUOLAR PROTEIN SORTING2.1 (VPS2.1) impaired autophagic degradation, as demonstrated by an overall accumulation of autophagic components (i.e. ATG8 and NBR1), and a reduced level of monodansylcadaverine (MDC)-labeled autophagic bodies in the vacuole (Katsiarimpa *et al.*, 2013). Similar autophagic defects were

observed by depletion of ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM 1 (AMSH1), which acts as AMSH3-related deubiquitinating enzyme and interacts with VPS2.1 (Katsiarimpa *et al.*, 2013). Loss of another ESCRT protein, CHARGED MULTIVESICULAR BODY PROTEIN 1 (CHMP1), results in delayed maturation/closure of phagophores, as well as aberrant plastid division, thereby linking ESCRT machinery to autophagic breakdown of plastid cargoes (Spitzer *et al.*, 2015). Additional molecular support arises from the characterization of FYVE DOMAIN PROTEIN REQUIRED FOR ENDOSOMAL SORTING 1 (FREE1, also known as FYVE1) as a plant-unique ESCRT component (Gao *et al.*, 2015; Kolb *et al.*, 2015; Gao *et al.*, 2014). Apart from its crucial role in the formation of both MVB/PVC and vacuoles, FREE1/FYVE1 has been implicated in the autophagic pathway through its direct interaction with SH3P2 and association with the PI3K complex (Gao *et al.*, 2015; Kolb *et al.*, 2015; Gao *et al.*, 2014).

In animals and yeast, the small GTPase Rab7 has been reported to play pivotal roles in the maturation of both autophagosomes and endosomes, and their subsequent fusion events with the lytic compartments (Hyttinen *et al.*, 2013). Notably, RabG3b, a homolog of Rab7 in Arabidopsis, co-localizes with the autophagosomal markers ATG8a and ATG8e (Kwon *et al.*, 2013; Kwon *et al.*, 2010). Furthermore, RabG3b was shown to modulate tracheary element (TE) differentiation and hypersensitive PCD via autophagy (Kwon *et al.*, 2013; Kwon *et al.*, 2010).

1.2.5 Role of autophagy in plant development and stress responses

Autophagy processes in plants have already been investigated in 1960s, but initial studies were limited to the morphological description using electron microscopic approaches. In recent years, genetic analyses (i.e. loss-of-function analysis of *ATG* genes) have significantly advanced our understanding of the molecular mechanisms and physiological functions of autophagy in plants.

Autophagy is activated in response to a wide range of abiotic stresses (Liu & Bassham, 2012; Thompson *et al.*, 2005; Hanaoka *et al.*, 2002). When autophagy-deficient plants grow under nitrogen- and carbon-depleted conditions, they typically show exaggerated starvation-triggered chlorosis and senescence, implying that nutrient remobilization in response to starvation requires autophagic activity (Suttangkakul *et al.*, 2011; Thompson *et al.*, 2005; Hanaoka *et al.*, 2002). Furthermore, oxidative stress, triggered for instance by treatment with H_2O_2 or methyl viologen, is known to activate autophagy (Xiong et al., 2007). Autophagy-deficient *AtATG18a*-RNAi Arabidopsis plants and *Osatg10b* rice mutants are hypersensitive to oxidative stress and accumulate more oxidized proteins, indicating that oxidized proteins are

removed by autophagy in plant cells (Shin *et al.*, 2009; Xiong *et al.*, 2007). Similarly, the autophagy pathway is induced and required for turnover of ER membranes in response to ER stress (Liu *et al.*, 2012). It has also been shown that autophagy is essential for plant tolerance against drought and high salinity stresses (Liu *et al.*, 2009), most likely because autophagy maintains cellular homeostasis by degrading aggregated or damaged proteins and organelles under these conditions.

There is emerging evidence for important roles of autophagy in phytohormone signaling and homeostasis. The defence hormone salicylic acid (SA) is known to induce autophagy (Yoshimoto *et al.*, 2009), and loss of function of *ATG* genes leads to accumulation of SA, which coincides with the onset of senescence (Yoshimoto *et al.*, 2009). Importantly, premature senescence and immunity-associated cell death phenotypes in *atg* mutants require a functional SA signaling pathway, which employs NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) as central signaling hub (Yoshimoto *et al.*, 2009). Based on these results, it was proposed that autophagy is involved in a negative feedback mechanism to suppress SA-dependent phenotypes. Moreover, the aforementioned autophagy cargo receptor TSPO can be induced by application with the stress-related hormone abscisic acid (ABA) (Vanhee *et al.*, 2011; Guillaumot *et al.*, 2009), suggesting a possible link between the autophagy machinery and plant responses to ABA. Finally, brassinosteroids (BRs) are linked to autophagic cell death associated with tracheary element (TE) differentiation (Kwon *et al.*, 2010). It was hypothesized that BRs might function as cell death signals to induce the autophagy pathway through activation of RabG3b, resulting in formation of the mature TE (Kwon *et al.*, 2010). Notably, a recent study suggests that BR-related transcription factor named BZR1 could be turned over through autophagy upon TOR inactivation, thus integrating autophagy into the BR signaling pathway (Zhang *et al.*, 2016b).

Due to potential pleiotropic effects caused by inactivation of autophagy, an alternative gain-of-function approach (e.g. overexpression of *ATG* genes) could be suitable to directly address the functions of autophagy in more detail.

1.3 Plant innate immunity

In order to fight against pathogen attack, plants have developed various sophisticated mechanisms to trigger defence responses that are constantly modulated. A 'zigzag' model of the long-lasting co-evolutionary struggle between plants and pathogens was proposed (Jones & Dangl, 2006). In this model, two major branches of the innate immune system have been defined:

PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) that are associated with different perception mechanisms in plants (Figure 3). Plasma membrane-resident pattern recognition receptors (PRRs) sense pathogen-associated molecular patterns (PAMPs) and induce a complex antimicrobial response known as PTI. PTI can be suppressed by pathogen-derived molecules, termed effectors, leading to effector-triggered susceptibility (ETS). Another type of immune receptors encoded by so-called resistance (*R*) genes monitors the presence or activity of such pathogen effectors, resulting in effector-triggered immunity (ETI).

1.3.1 PAMP-triggered immunity (PTI)

PAMPs, also known as microbe-associated molecular patterns (MAMPs), are highly conserved molecular motifs present in a whole class of microbes (Boller & Felix, 2009; Nurnberger & Brunner, 2002). At the frontline of innate immunity, plants utilize surface PRRs, which are receptor-like kinases (RLKs) or receptor-like proteins (RLPs), to perceive PAMPs (Newman *et al.*, 2013).

One of the best-characterized PRRs is the leucine-rich repeat RLK (LRR-RLK) FLAGELLIN-SENSITIVE 2 (FLS2) from Arabidopsis, which recognizes the bacterial PAMP flagellin or the flagellin-derived peptide flg22 (Chinchilla *et al.*, 2006; Gomez-Gomez & Boller, 2000). Ligand binding to FLS2 triggers complex formation and phosphorylation events between FLS2 and the RLK BRI1-ASSOCIATED KINASE 1 [BAK1, also called SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3 (SERK3)], and result in the activation of complex downstream signaling and defence responses (Figure 3) (Boller & Felix, 2009). Non-activated FLS2 receptors constitutively follow the endosomal recycling pathway which is sensitive to the trafficking inhibitor BFA (Beck *et al.*, 2012). By contrast, upon flg22 stimulation, activated FLS2 receptors traffic via a BFA-insensitive pathway and are delivered to the MVB/PVC followed by subsequent degradation in the vacuole (Figure 3) (Beck *et al.*, 2012). Hence, vacuolar turnover of ligand-activated FLS2 contributes to the regulation of the plasma membrane pool of FLS2 by quenching receptor activities.

Other PAMP-PRR pairs involved in PTI include the bacterial elongation factor Tu (EF-Tu) sensed by EF-TU RECEPTOR (EFR) (Zipfel *et al.*, 2006; Kunze *et al.*, 2004), bacterial peptidoglycan (PGN) detected by the LYM1 LYM3 CERK1 PGN perception system (Willmann *et al.*, 2011), fungal chitin recognized by CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (Miya *et al.*, 2007), and lipopolysaccharide perceived by the recently identified RLK LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE) (Ranf *et al.*, 2015).

PAMP/PRR-triggered PTI responses typically involve the accumulation of reactive oxygen species (ROS) (also known as oxidative burst), activation of mitogen-activated protein kinase (MAPK) cascades, stomatal closure, large-scale reprogramming of gene expression, callose deposition, and the production of antimicrobial secondary metabolites (Boller & Felix, 2009).

1.3.2 Effector-triggered immunity (ETI)

Successful pathogens deploy multiple virulence factors, called effectors, into plant cells to promote virulence. Pathogen effectors target host proteins to interfere with PTI and to manipulate physiological processes for the benefit of infection (Bent & Mackey, 2007). Plants, in turn, have evolved a second tier of defence based on intracellular resistance (R) proteins containing nucleotide-binding (NB) and leucine-rich repeat (LRR) domains (Figure 3) (Dangl & Jones, 2001). After direct or indirect recognition of effectors, plant NB-LRR immune receptors become activated, resulting in ETI with faster and stronger defence reactions than PTI. ETI often culminates in a programmed cell death (PCD) reaction at the site of pathogen entry, known as the hypersensitive response (HR) (Coll *et al.*, 2011; Jones & Dangl, 2006; Greenberg & Yao, 2004), and the induction of systemic acquired resistance (SAR) responses in non-infected distal parts of the plant (Fu & Dong, 2013).

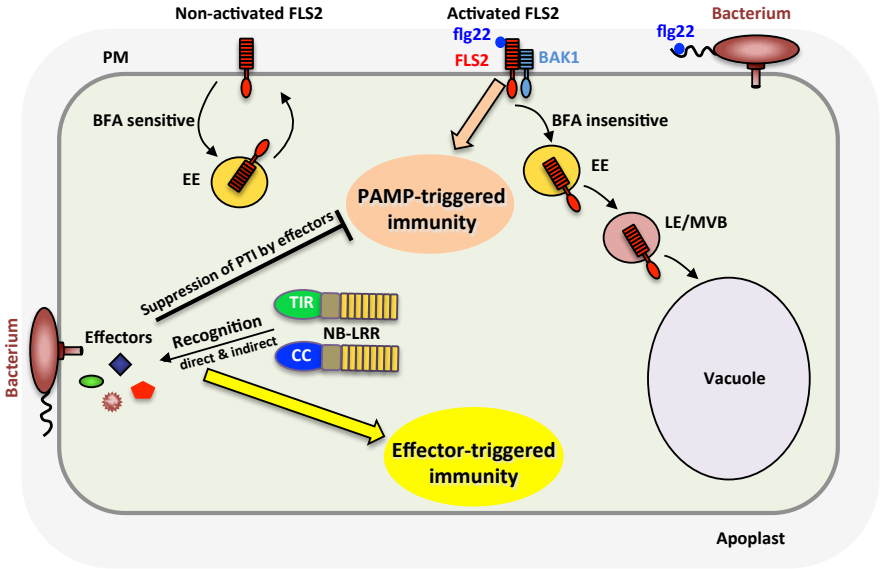

Figure 3. PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). Plasma membrane-resident PRRs sense PAMPs and induce a complex antimicrobial response known as PTI. One of the best-characterized PRRs is FLS2, which recognizes the bacterial PAMP flagellin or the flagellin-derived peptide flg22. Non-activated FLS2 receptors constitutively follow the

recycling endosomal pathway which is sensitive to the trafficking inhibitor BFA. Ligand flg22 binding to FLS2 triggers complex formation and phosphorylation events between FLS2 and BAK1, which leads to activation of the PTI response. Activated FLS2 receptors traffic via a BFA-insensitive pathway and are delivered to the MVB/PVC followed by subsequent vacuolar degradation. Successful pathogens deploy multiple effectors into plant cells to interfere with PTI. Plants utilize intracellular immune receptors containing nucleotide-binding (NB) and leucine-rich repeat (LRR) domains to recognize effectors either directly or indirectly, resulting in ETI.

Based on the presence of different N-terminal domains, NB-LRR R proteins can be further categorized into two subgroups: the Toll/Interleukin-1 receptor (TIR)- or coiled-coil (CC)-domain containing NB-LRR proteins (Meyers *et al.*, 1999). The molecular mechanisms and downstream events that follow NB-LRR activation are still not fully understood. However, it is well-established that the downstream signaling components required for ETI are different for the two NB-LRR classes. TIR-NB-LRR-type immune receptors typically engage the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)/PHYTOALEXIN DEFICIENT 4 (PAD4)/SENESCENCE-ASSOCIATED GENE 101 (SAG101) complex to mount a defence response, whereas signaling activated by CC-NB-LRR immune receptors usually depends on NONRACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) (Zhu *et al.*, 2011; Feys *et al.*, 2005; Aarts *et al.*, 1998). It has also been shown that certain pathogen effectors are recognized by both subclasses of NB-LRR proteins and could thereby stimulate NDR1- and EDS1-dependent signal transduction in parallel to generate a full immune response (Eitas *et al.*, 2008).

Several models for the recognition of pathogen effectors by R proteins have been proposed, including the guard hypothesis and the decoy model (van der Hoorn & Kamoun, 2008; Jones & Dangl, 2006). The guard hypothesis proposes that R proteins (‘guards’) monitor the activities of pathogen effectors on host proteins (‘guardees’) due to their roles in defence responses or as susceptibility factors. One of the most extensively studied examples for the guard hypothesis engages two CC-NB-LRR R proteins, RESISTANCE TO P. SYRINGAE PV MACULICOLA 1 (RPM1) and RESISTANCE TO P. SYRINGAE 2 (RPS2), which are associated with the Arabidopsis host protein RPM1-INTERACTING PROTEIN 4 (RIN4) (Kim *et al.*, 2005; Axtell & Staskawicz, 2003; Mackey *et al.*, 2003). RPM1 senses the hyperphosphorylation of RIN4 induced by effectors AvrRpm1 and AvrB, whereas RPS2 perceives AvrRpt2-mediated RIN4 cleavage, leading in both cases to ETI (Mackey *et al.*, 2003; Mackey *et al.*, 2002). RIN4 has recently been linked to the modulation of immune system outputs by acting as a “phosphoswitch” (Chung *et al.*, 2014). Upon flagellin recognition, RIN4 is phosphorylated on serine 141, which de-represses various immune responses.

An alternative hypothesis, the decoy model, suggests that the host decoy protein mimics the actual effector target (guardee) and serves as a "bait" to trap effectors, thus triggering immune responses (Collier & Moffett, 2009; van der Hoorn & Kamoun, 2008). In striking contrast to guardee proteins, decoys do not play a role in host defence or susceptibility when the cognate R protein is absent (van der Hoorn & Kamoun, 2008). Detailed molecular support for the decoy model could be derived from the indirect recognition of the bacterial protease effector AvrPphB by the Arabidopsis CC-NB-LRR protein RESISTANCE TO P. SYRINGAE 5 (RPS5) (Ade *et al.*, 2007). RPS5-mediated detection of AvrPphB requires the decoy protein kinase AVRPPHB SUSCEPTIBLE 1 (PBS1) (Ade *et al.*, 2007). AvrPphB-induced proteolytic cleavage of PBS1 leads to the conformational change in PBS1, which is necessary for RPS5 activation (DeYoung *et al.*, 2012). Such conformational change can also be induced by a five-amino-acid insertion at the cleavage site of PBS1 protein and results in RPS5 activation in the absence of effectors (DeYoung *et al.*, 2012). In theory, RPS5 could be able to detect any pathogen-derived effector that induces the requisite conformational change in PBS1. Recently, the recognition specificity of RPS5 was successfully altered by exchanging the AvrPphB cleavage site in PBS1 with the cleavage sequence targeted by unrelated pathogen-secreted proteases (Kim *et al.*, 2016). Hence, the engineering of decoys to expand recognition specificities of resistance proteins provides novel opportunities for breeding of pathogen-resistant crops.

Recent advances in the understanding of immune receptor function include the emerging recognition of complementary NB-LRR pairs that are required to detect effectors from a single or even from multiple pathogens (Saucet *et al.*, 2015; Eitas & Dangl, 2010). Intriguingly, the characterization of the RPS4/RRS1 NB-LRR pair revealed that the recognition of bacterial and fungal effectors (i.e. AvrRps4, Pop2) is mediated by their binding to a WRKY domain present in RRS1. Based on the important role of WRKY transcription factors in the activation of defence responses, an "integrated decoy" model for direct recognition of pathogen effectors has been proposed (Le Roux *et al.*, 2015; Sarris *et al.*, 2015; Cesari *et al.*, 2014).

1.3.3 Autophagy in plant immunity

Recent years have seen tremendous progress in unraveling the mechanistic role for plant autophagy in pathological situations.

Firstly, autophagy has been implicated in regulation of HR cell death induced by avirulent strains of different pathogens. Genetic inactivation/suppression of *ATG* genes leads to a gradual spread of cell death far beyond the primary HR lesions after infection with avirulent virus and

bacterial strains, suggesting that autophagy is required for restricting immunity-associated cell death (Yoshimoto *et al.*, 2009; Patel & Dinesh-Kumar, 2008; Liu *et al.*, 2005). A number of studies also demonstrated a death-promoting function of autophagy during HR (Coll *et al.*, 2014; Hackenberg *et al.*, 2013; Kwon *et al.*, 2013; Hofius *et al.*, 2009). In particular, autophagy was found to contribute to HR cell death mediated by the TIR-type NB-LRR protein RPS4 and CC-type NB-LRR protein RPM1 (Hofius *et al.*, 2009). Such HR-promoting autophagic cell death seems to be NPR1-independent (Minina *et al.*, 2014; Munch *et al.*, 2014). In contrast, tissue collapse and cell death observed in old *atg* mutants several days after infection rely on NPR1 (Yoshimoto *et al.*, 2009) and might be caused by enhanced ER stress in response to autophagy-deficient conditions (Minina *et al.*, 2014; Munch *et al.*, 2014). Therefore, these two types of cell death can be separated genetically and temporally. To directly address the effect of autophagy in the induction of HR, it would be important to use alternative gain-of-function approaches. Indeed, a positive role of autophagy in HR induction was further supported by transgenic expression of a constitutively active version of the Rab GTPase RabG3b, which resulted in enhanced autophagy levels and accelerated HR cell death (Kwon *et al.*, 2013).

Secondly, autophagy is known to modulate plant defence in response to hemibiotrophic pathogens. For instance, Lenz and co-workers performed comprehensive analyses to determine resistance characteristics of autophagy-deficient mutants to virulent *Pst* DC3000 (Lenz *et al.*, 2011). This study revealed that *atg* mutants are more resistant towards virulent *Pst* DC3000, which does not seem to be caused by altered PTI responses. Notably, the basal SA levels in *atg* mutants are slightly but significantly increased compared to wild-type. This difference is even more pronounced upon pathogen infection and results in stronger up-regulation of SA-inducible gene expression and camalexin production in *atg* mutants relative to wild-type. Hence, augmented SA levels in *atg* mutants seem to be responsible for enhanced resistance to virulent *Pst* DC3000. The negative role of autophagy in SA-associated plant immunity has been independently confirmed by the finding that loss of ATG5 confers resistance to virulent *Pst* DC3000, while overexpression of a constitutively active version of RabG3b leads to stimulation of autophagy and increased susceptibility (Kwon *et al.*, 2013). Recently, the effector protein PexRD54 from the hemibiotrophic oomycete pathogen, *Phytophthora infestans*, was reported to target autophagy-related processes in plant cells (Dagdas *et al.*, 2016). More specifically, PexRD54 can interact with ATG8CL (belonging to potato ATG8 family) via its AIM/LIR domain and promote autophagosome formation. Furthermore, PexRD54 out-competes the tobacco

NBR1 homologue JOKA2 from ATG8CL-labeled autophagosomes to suppress NBR1/JOKA2-mediated disease resistance. These findings indicate that pathogens have evolved effector-based strategies to manipulate or even hijack autophagy pathways to promote virulence. It remains on open question whether hemibiotrophic bacterial pathogens would benefit from increased plant autophagy levels and thus might have evolved effectors with similar autophagy-stimulating activities as PexRD54.

Thirdly, autophagy-defective mutants were shown to exhibit enhanced cell death and resistance in response to the powdery mildew fungus *Golovinomyces cichoracearum*, an obligate biotrophic pathogen (Wang *et al.*, 2011b; Wang *et al.*, 2011c). Interestingly, inactivation of SA signaling fully suppressed powdery mildew resistance in *atg2* but only partially alleviated the cell death phenotype, indicating that cell death can be uncoupled from disease resistance (Wang *et al.*, 2011c). Likewise, *amsh1* mutants defective in autophagic degradation were shown to be more resistant to powdery mildew infection and show induction of SA marker gene expression (Katsiarimpa *et al.*, 2013).

Fourthly, several studies demonstrated that autophagy is required for plant resistance to necrotrophic fungal pathogens. Induction of *ATG* gene expression and increased accumulation of GFP-ATG8-marked autophagosomal structures indicated activation of autophagy upon infection with *Botrytis cinerea* (Lai *et al.*, 2011). Moreover, autophagy-deficient mutants (i.e. *atg* mutants and *amsh1* mutants) showed enhanced susceptibility to the necrotrophic pathogens *Botrytis cinerea* and *Alternaria brassicicola* (Katsiarimpa *et al.*, 2013; Lai *et al.*, 2011; Lenz *et al.*, 2011). The potential mechanisms by which autophagy promotes disease resistance against necrotrophic pathogens have recently been discussed (Zhou *et al.*, 2014). Necrotrophic pathogens kill host cells during the initial stage of infection and benefit from plant cell death. Rapidly activated autophagy might suppress pathogen-triggered cell death by clearance of damaged and toxic cellular contents, thereby contributing to resistance. Modulation of SA and jasmonate (JA) signaling pathways by autophagy might function as an alternative mechanism. Inactivation of autophagy was shown to result in higher steady-state transcript levels of *PDF1.2* and *PR1*, which are regulated by JA and SA, respectively (Lai *et al.*, 2011). However, upon *Botrytis* infection, only *PR1* expression remained substantially higher whereas JA-regulated *PFD1.2* levels were significantly decreased in *atg* mutants compared to wild-type (Lai *et al.*, 2011). Hence, autophagy appears to positively regulate JA-mediated plant defence responses, which are known to act mainly against necrotrophic pathogens (Mengiste, 2012).

Lastly, autophagy has been shown to play a crucial role in host-virus interactions. It is generally accepted that RNA silencing triggered by viral

double-stranded RNA (dsRNA) serves as an antiviral defence mechanism in plants. The viral silencing suppressor P0 from polerovirus co-opts autophagy for selective degradation of ARGONAUTE1 (AGO1), which has a central role in RNA silencing, thus counteracting host defence (Derrien *et al.*, 2012). Another study suggests that autophagy is hijacked for propagation of a DNA virus in infected algae (Schatz *et al.*, 2014). This study has shown that autophagy is activated upon viral infection and blocking autophagy by a pharmacological approach resulted in prominent reduction in viral release (Schatz *et al.*, 2014). Importantly, the host-derived ATG8-PE conjugate could be detected from purified virions (Schatz *et al.*, 2014). In contrast to the potential proviral roles of the autophagy pathway, some studies might also point to the contribution of autophagy to antiviral defence responses. For instance, the tobacco rgs-CaM, a calmodulin-like protein, was demonstrated to bind to viral suppressors of RNA silencing in order to both interfere with suppressor activity and facilitate their degradation via the autophagy pathway (Nakahara *et al.*, 2012). A recent study revealed that the silencing suppressor P6 from *Cauliflower mosaic virus* (CaMV) is able to suppress SA-triggered autophagy through binding and activation of the Arabidopsis target-of-rapamycin (TOR) (Zvereva *et al.*, 2016), which resulted in suppression of antibacterial defences. However, the consequences of altered autophagy levels on CaMV infection remain to be determined.

1.4 Plant cell death

Programmed cell death (PCD) plays a number of fundamental roles throughout the plant life, including xylogenesis, embryo and leaf development, various stages of plant reproduction, senescence, as well as defence responses to abiotic and biotic stresses (Hofius *et al.*, 2007; Greenberg, 1996). In animals, PCD has been categorized into three major types: apoptosis, autophagic cell death, and necrosis (Lockshin & Zakeri, 2004). Plants have also evolved different mechanisms and pathways for cell demise, which show similarities but also substantial differences in comparison to their counterparts in animals and other eukaryotic organisms (Bozhkov & Lam, 2011; van Doorn *et al.*, 2011).

1.4.1 Classification of PCD in plants

In contrast to animal cells, classic apoptosis is absent in plants (van Doorn *et al.*, 2011). Based on morphological criteria, two major classes of cell death have been proposed: vacuolar and necrotic cell death (van Doorn *et al.*, 2011). Execution of vacuolar cell death typically involves both autophagy and a

massive release of hydrolases by vacuole collapse, leading to complete removal of cellular contents. Necrosis is defined by cytological hallmarks including mitochondrial swelling, early rupture of plasma membrane, protoplast shrinkage, and absence of vacuolar PCD features.

Intriguingly, HR-associated cell death conditioned by R protein activation could not be assigned to either type, and was considered to be a mixed form of cell death for the following reasons (van Doorn *et al.*, 2011). Firstly, HR PCD often displays features of necrotic cell death (van Doorn *et al.*, 2011). Secondly, HR is usually associated with the expansion of vacuoles and subsequent rupture of the tonoplast, which in certain instances could engage vacuole-resident VACUOLAR PROCESSING ENZYME (VPE) (van Doorn *et al.*, 2011; Hatsugai *et al.*, 2004; Rojo *et al.*, 2004). Thirdly, it has been demonstrated that autophagy is activated during HR and that some forms of HR triggered by TIR- and CC-type of NB-LRR proteins require autophagic components (Hofius *et al.*, 2009). Lastly, in striking contrast to vacuolar cell death, HR-associated vacuolar collapse does not seem to result in complete removal of the protoplast (van Doorn *et al.*, 2011; Beers & McDowell, 2001). Cytological characteristics of PCD implicated in HR can differ. For example, HR mediated by CC-NB-LRR immune receptors RPM1 and RPS2 is dependent on membrane fusion between the plasma membrane and the tonoplast, which leads to the release of vacuolar defence contents into the extracellular space where bacteria proliferate (Hatsugai *et al.*, 2009). In contrast, virus-triggered HR cell death conditioned by the TIR-NB-LRR N protein involves VPE activity, and requires collapse of vacuoles and the release of hydrolases to restrict virus proliferation within cells (Hatsugai *et al.*, 2004).

1.4.2 Lesion mimic mutants and cell death suppressors

One fundamental approach to decipher HR and defence pathways is the exploitation of mutants that exhibit accelerated cell death (acd) and activated immune responses in the absence of effectors and pathogen infection (Moeder & Yoshioka, 2008; Lorrain *et al.*, 2003). These lesion mimic mutants provide a valuable resource to identify novel players in HR cell death and to further dissect underlying signaling pathways (Moeder & Yoshioka, 2008; Lorrain *et al.*, 2003).

The *lesion simulating disease resistance 1* (*lsd1*) mutant exhibits a so-called runaway cell death phenotype, meaning that HR occurs normally but the spread of cell death can not be subsequently controlled (Dietrich *et al.*, 1994). Such cell death phenotype relies on signaling components required for pathogen recognition and accumulation of the defence hormone SA (Aviv *et al.*, 2002; Rusterucci *et al.*, 2001; Jabs *et al.*, 1996). Importantly, loss of

function of the CC-NB-LRR protein ACTIVATED DISEASE RESISTANCE1-LIKE2 (ADR1-L2) suppressed *lsd1* runaway cell death (Bonardi *et al.*, 2011). Moreover, the metacaspase AtMC1 functions as a positive regulator of runaway cell death in *lsd1*, whereas AtMC2 is a negative regulator of AtMC1 (Coll *et al.*, 2010). Importantly, it has recently been demonstrated that AtMC1 and autophagy act in separate pathways to modulate HR cell death and senescence (Coll *et al.*, 2014).

Several lesion mimic mutants, including *acd5* and *acd11*, were shown to be disrupted in genes associated with sphingolipid metabolism and signaling (Bruggeman *et al.*, 2015). *ACD5* encodes a ceramide kinase that possesses high specificity to ceramides but not other sphingolipids *in vitro* (Liang *et al.*, 2003). It has been proposed that the dynamic balance between free and phosphorylated ceramides plays a critical role in the modulation of plant PCD (Liang *et al.*, 2003). *acd5* mutants show spontaneous cell death late during development, which is associated with ceramide accumulation, autophagy induction, and mitochondrial ROS accumulation (Bi *et al.*, 2014; Liang *et al.*, 2003). In addition, *acd5* mutant plants exhibit enhanced susceptibility to *Pseudomonas syringae* and *Botrytis cinerea* (Bi *et al.*, 2014; Greenberg *et al.*, 2000).

The lethal, recessive *acd11* mutant shows autoimmune cell death and constitutive activation of defence responses due to disruption of a ceramide-1-phosphate transfer protein (Simanshu *et al.*, 2014; Brodersen *et al.*, 2002). In *acd11*, normally low ceramide-1-phosphate levels are increased, but relatively abundant phytoceramides rise acutely, supporting the previous notion that changes in the distribution and balance of these two sphingolipids are essential for cell death regulation in plants (Simanshu *et al.*, 2014). PCD in *acd11* is initiated at an early seedling stage and was shown to be dependent on SA and the EDS1-dependent signaling pathway. *acd11* autoimmunity can be fully suppressed by transgene introgression of the bacterial SA hydroxylase gene *NahG* (Brodersen *et al.*, 2005) and restored by application of the functional SA analog, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Malinovsky *et al.*, 2010; Gorlach *et al.*, 1996). Based on these features, mutagenized *acd11 NahG* populations were previously used to screen for *lazarus* (*laz*) suppressors of BTH-triggered cell death. Map-based cloning analyses revealed that *acd11* autoimmunity requires the histone lysine methyltransferase LAZ2, as well as the TIR-NB-LRR protein LAZ5, whose expression is dependent on LAZ2 activity (Palma *et al.*, 2010). These finding suggested that autoimmune cell death in *acd11* mimics the HR and is a consequence of ectopic activation of LAZ5. Furthermore, *LAZ1* was found to encode a six-transmembrane protein containing a domain of unknown function

(DUF300) (Malinovsky *et al.*, 2010). LAZ1 shares sequence homology and structural similarity with the family of DUF300 proteins implicated in tumor suppression, post-Golgi membrane trafficking, and steroid transport in animals (Olivier-Mason *et al.*, 2013; Dawson *et al.*, 2010; Malinovsky *et al.*, 2010; Best & Adams, 2009; Best *et al.*, 2008; Akaishi *et al.*, 2007). In addition to its role in *acd11* autoimmunity, characterization of an independent *laz1* T-DNA insertion mutant (*laz1-5*) pointed to LAZ1 functions in fully or partly autophagy-dependent HR conditioned by RPS4 and RPM1, respectively (Malinovsky *et al.*, 2010; Hofius *et al.*, 2009). Based on these findings, the characterization of *acd11* suppressors might reveal novel genes and pathways required for regulation of immune receptor function, autophagy, cell death, and disease resistance.

1.5 Brassinosteroid signaling

Brassinosteroids (BRs) act as essential phytohormones in plant growth and development and in response to different environmental cues. Loss-of-function mutants that are impaired in BR perception or biosynthesis often display a strong dwarf phenotype associated with compromised photomorphogenesis and altered fertility (Clouse & Sasse, 1998). In contrast to steroid signaling in animals that relies on intracellular receptors, BR signaling in plants is predominantly mediated by cell surface-localized receptors BRI1, BRI1-LIKE1 (BRL1), BRL3, and also by the co-receptor BAK1/SERK3 and other SERKs (Gou *et al.*, 2012; Cano-Delgado *et al.*, 2004; Li & Chory, 1997). BRs are perceived by the BRI1 receptor at the PM, which subsequently triggers an intracellular signal transduction cascade that modulates transcriptional responses (Zhu *et al.*, 2013).

1.5.1 BR signal transduction

The kinase activity of BRI1 is maintained at basal level by two inhibitory mechanisms: the auto-inhibitory effect of its own C-terminal tail, as well as the direct interaction between the BRI1 kinase domain and a negative regulator of BR signaling, BRI1 KINASE INHIBITOR 1 (BKI1) (Wang & Chory, 2006; Wang *et al.*, 2005). Binding of BRs to the BRI1 extracellular domain (ECD) triggers its kinase activity (Kim & Wang, 2010). The mode of action in BRI1 activation is known to involve the release of phosphorylated BKI1 from the plasma membrane into the cytosol, the recruitment of its co-receptor BAK1/SERK3 for receptor heterooligomer formation, and sequential transphosphorylation events within the receptor/co-receptor complex (Gou *et al.*, 2012; Clouse, 2011; Jaillais *et al.*, 2011; Wang *et al.*, 2008; Wang &

Chory, 2006; Li *et al.*, 2002; Nam & Li, 2002). Interestingly, it has also been suggested that phosphorylated BKI1 functions as positive regulator of BR signaling pathway via interaction with 14-3-3 family members (Wang *et al.*, 2011a).

Upon successful formation of the BR receptor/co-receptor complex on the plasma membrane, BRI1 subsequently phosphorylates several receptor-like cytoplasmic kinase (RLCK) proteins including BR-SIGNALING KINASE1 (BSK1) and CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) (Kim *et al.*, 2011; Tang *et al.*, 2008). The activation of BSK1 and CDG1 via BRI1 induces phosphorylation of the nucleocytoplasmic phosphatase called BRI1 SUPPRESSOR 1 (BSU1) (Kim *et al.*, 2011; Kim *et al.*, 2009). In turn, phosphorylated BSU1 was shown to dephosphorylate BRASSINOSTEROID-INSENSITIVE 2 (BIN2) at the conserved phospho-Tyr200 site and thus to suppress the kinase activity of BIN2 (Kim *et al.*, 2009). BIN2 negatively regulates the BR signaling pathway by phosphorylating a group of transcription factors including BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1, also known as BZR2) (Wang *et al.*, 2002; Yin *et al.*, 2002). Furthermore, phosphorylated BZR1 and BES1/BZR2 with abolished DNA-binding activity show 14-3-3 protein-mediated cytoplasmic retention (Figure 4) (Gampala *et al.*, 2007; Vert & Chory, 2006). Following inactivation of BIN2, these two transcription factors are thought to be dephosphorylated by PROTEIN PHOSPHATASE 2A (PP2A) and redistributed into the nucleus to modulate the expression of BR-responsive genes (Figure 4) (Tang *et al.*, 2011; Kim & Wang, 2010).

1.5.2 Subcellular regulation of BR signaling

Over the last years, a lot of knowledge has been gained on the molecular components and regulatory mechanisms involved in the BR signaling pathway. However, the interplay between BR signaling and membrane trafficking of the BR receptor and co-receptor is still not well understood. Several studies have revealed that BRI1 is not only localized to the plasma membrane but also to the TGN/EE, MVB/PVC, and vacuolar lumen (Viotti *et al.*, 2010; Geldner *et al.*, 2007; Dettmer *et al.*, 2006; Russinova *et al.*, 2004). Furthermore, BRI1 constantly cycles between the plasma membrane and the TGN/EE, and it is delivered for vacuolar turnover via the MVB/PVC in a ligand-independent manner (Figure 4) (Viotti *et al.*, 2010; Geldner *et al.*, 2007; Russinova *et al.*, 2004). The trafficking inhibitor BFA was shown to enhance BRI1 endosomal accumulation and activate the BR pathway, indicating that endosomes likely act as platform for BR signaling (Geldner *et al.*, 2007). However, it was reported that BR signaling could only be activated by BFA inhibition of ARF-

GEF-mediated BRI1 endocytosis, and not by enhanced BRI accumulation in endosomal compartments (Irani *et al.*, 2012). Consistent with this finding, the increase of PM pools of BRI1 via genetic or pharmacological approaches resulted in activation of BR signaling (Irani *et al.*, 2012).

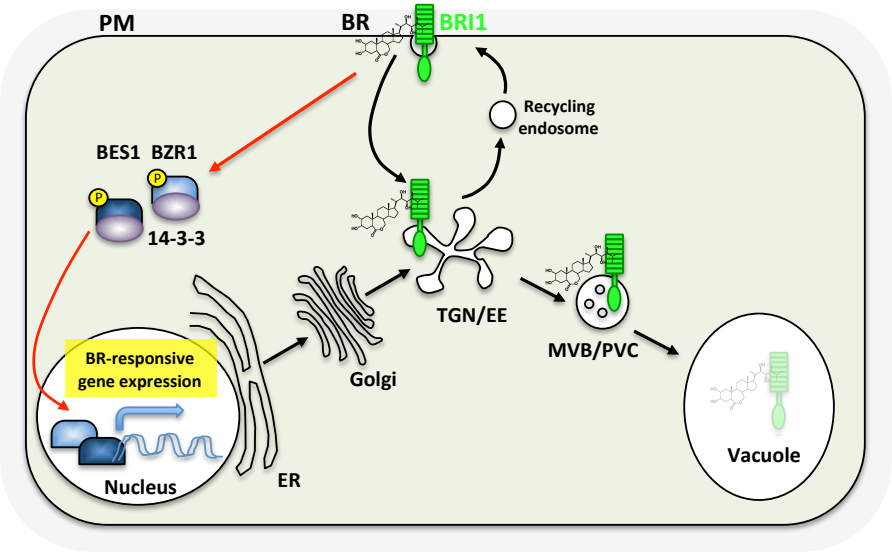

Figure 4. Schematic diagram presenting BRI1 trafficking and certain downstream events that follow activation of BR signaling. Endocytosed BRI1 receptors are sorted at the TGN/EE to be either recycled back to the cell surface or transported for vacuolar turnover via the MVB/PVC in a ligand-independent manner. The trafficking of BRI1-ligand complex has been successfully visualized with the development of Alexa Fluor 647-coupled castasterone (AFCS), which functions as a fluorescent ligand of BRI1 (Irani *et al.*, 2012). In particular, accumulation of AFCS was observed in the vacuolar lumen (Irani *et al.*, 2012), which implies the possibility that as yet uncharacterized vacuolar pool of BRs may be important for the regulation of intracellular BRs levels and homeostasis. Phosphorylated transcription factors BZR1 and BES1 show cytoplasmic retention mediated by 14-3-3 proteins. Upon activation of BR signaling, these two transcription factors are dephosphorylated and redistributed into the nucleus to modulate the expression of BR-responsive genes.

Growing evidence supports the notion that BRI1 signals mainly from the plasma membrane. Endocytosis of BRI1 was perturbed by inactivation of ADAPTOR PROTEIN COMPLEX-2 (AP-2), resulting in activated BR signaling (Di Rubbo *et al.*, 2013). Furthermore, impaired acidification of the TGN/EE in *deetiolated3* (*det3*), a V-ATPase mutant, causes reduced exocytosis and recycling of BRI1, thereby rendering this mutant insensitive to exogenous BR treatment (Luo *et al.*, 2015). Importantly, a recent study suggested that ubiquitination of BRI regulates its internalization, endosomal recycling, and vacuolar degradation (Martins *et al.*, 2015).

It has previously been proposed that BRI1 trafficking is influenced by its co-receptor BAK1 (Russinova *et al.*, 2004). Using a protoplast system, transient co-overexpression of BRI1 along with BAK1 causes enhanced endocytosis and vacuolar degradation of BRI1 (Russinova *et al.*, 2004). Furthermore, the visualization of receptor/co-receptor heterooligomers implied that BR signal transduction engages interaction of BRI1 and BAK1 in pre-assembled complexes at the plasma membrane, and a relatively small portion of the complexes is utilized by BR signaling (Bucherl *et al.*, 2013). In this study, BAK1 was surprisingly found on the tonoplast, raising the possibility that this uncharacterized intracellular pool of BAK1 contributes to the regulation of BR signaling (Bucherl *et al.*, 2013).

1.5.3 The roles of BRs

BRI1 signaling is one of the best-characterized receptor kinase pathways in plants. Previous studies have indicated the interplay between BR/BRI1 and other well-studied receptor kinases. BAK1 functions as a co-receptor for both BRI1 and the immunity-associated receptor kinase FLS2. Therefore, sharing of the co-receptor BAK1 (in particular when BAK1 amount is limiting) was proposed to generate antagonism between BR and PTI responses (Belkhadir *et al.*, 2012; Wang, 2012). In contrast, another independent study showed that BAK1 is not rate-limiting and BR-mediated suppression of FLS2 pathway occurs independently of the complex FLS2-BAK1-BOTRYTIS-INDUCED KINASE1 (BIK1) (Albrecht *et al.*, 2012). The BR-related transcription factor BZR1 was recently shown to be a master regulator of the trade-off between growth and immunity in plants (Lozano-Duran & Zipfel, 2015; Lozano-Duran *et al.*, 2013).

It is well known that BRs play an important role in light regulation of plant growth and development (Li *et al.*, 1996; Szekeres *et al.*, 1996). Several studies have revealed the mechanisms underlying the crosstalk between BR and light pathways. BZR1 was demonstrated to interact with the light-regulated transcription factor PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), which facilitates plant growth co-regulation by BRs and light signals through the control of numerous common genomic targets (Oh *et al.*, 2012). Moreover, dark-induced and proteasome-dependent removal of the phosphorylated (inactive) form of BZR1 by an E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) enhances BR signaling (Kim *et al.*, 2014). Another key transcription factor in the light signaling pathway, ELONGATED HYPOCOTYL 5 (HY5), was recently shown to interact with BZR1 to regulate cotyledon opening (Li & He, 2016).

Substantial evidence indicates interaction between BRs and other hormonal signaling pathways. For instance, BZR1 directly regulates expressions of a large number of auxin-related genes (Sun *et al.*, 2010). The transcription factors BES1/BZR2 and AUXIN RESPONSE FACTOR 5 [ARF5, also called MONOPTEROS (MP)] are able to bind to the same promoter region required for BRs/auxin responses (Walcher & Nemhauser, 2012). Furthermore, it has been suggested that auxin induces BR biosynthetic gene expression and stimulates BR biosynthesis (Chung *et al.*, 2011; Yoshimitsu *et al.*, 2011). Importantly, phosphorylation and inactivation of AUXIN RESPONSE FACTOR 2 (ARF2) via BIN2 kinase have also been proposed to integrate BRs and auxin pathways (Vert *et al.*, 2008). In addition, the actin cytoskeleton appears to function as another convergence point of BR signaling and auxin responses (Lanza *et al.*, 2012).

Apart from the direct interplay with other signaling pathways, BRs also control diverse developmental processes, such as root development, male fertility, and flowering time. BR treatment seems to influence root growth in a dose-dependent manner, as it is promoted by low but suppressed by high concentrations (Mussig *et al.*, 2003). Likewise, both enhancement and inactivation of BR signaling lead to smaller root meristems and impaired root growth, indicating that optimal root development requires balanced BR signaling (Gonzalez-Garcia *et al.*, 2011). Therefore, these findings reveal that BR levels and BR signaling are tightly controlled, and imbalances in BR levels and/or BR signaling lead to defects in root growth. Furthermore, BRs are known to exert effects on meristem size in roots by regulating cell cycle activity (Gonzalez-Garcia *et al.*, 2011; Hacham *et al.*, 2011). It has been shown that BR mutants exhibit defects in male fertility, which coincides with decreased expression of multiple important genes involved in pollen and anther development (Ye *et al.*, 2010). Finally, BR-related mutants display late-flowering phenotypes, which could be, at least in part, explained by elevated expression of *FLOWERING LOCUS C* (*FLC*) (Domagalska *et al.*, 2007). RELATIVE OF EARLY FLOWERING 6 (REF6), which represses *FLC* expression, interacts with BES1/BZR2, and thus might point to a potential molecular mechanism for BR regulation of flowering time (Yu *et al.*, 2008; Noh *et al.*, 2004).

In addition to their crucial role in the trade-off between immunity and growth, the protective activity of BRs against disease both in tobacco and rice has been reported. However, the molecular basis for these effects remains unclear (Nakashita *et al.*, 2003). Furthermore, it was proposed that BRs may act as cell death signals to trigger the autophagy pathway during tracheary element differentiation (Kwon *et al.*, 2010). In apparent agreement with a

death-promoting function, BRs have been shown to induce apoptosis in prostate cancer cells, and therefore could be considered as promising anticancer drugs (Steigerova *et al.*, 2012). A major challenge is therefore to unravel the potential role of BR signaling in the context of immunity, cell death, and autophagy.

2 Aims of the study

Vacuoles are multifunctional and highly dynamic organelles that respond to cellular signals and environmental cues to regulate plant growth and development. In addition, several vacuolar defence strategies have been identified in plants. The overall goal of the thesis was to dissect the regulatory mechanisms underlying vacuole-mediated control of plant growth and immunity. More specifically, I aimed to understand the regulators and individual pathways involved in these mechanisms, as well as the crosstalk between different pathways. I also attempted to describe the physiological consequences and potential application of a genetically enhanced vacuolar trafficking pathway (i.e. autophagy) in plants. In this thesis work, we focused on the functional analyses of two *LAZARUS* (*LAZ*) genes and the characterization of plants constitutively overexpressing autophagy-related *ATG* genes.

The following specific aims were addressed:

- Investigate the role of the retromer complex in immunity-related autophagy and cell death.
- Analyze LAZ1 function in the regulation of immunity-associated autophagy.
- Characterize LAZ1-related DUF300 proteins in brassinosteroid signaling.
- Study the effect of overexpression of *ATG* genes on plant productivity and stress tolerance.

3 Results and Discussion

3.1 Retromer regulates hypersensitive cell death and autophagic degradation (Paper I)

Membrane trafficking plays a pivotal role in plant innate immunity and mediates activation of immune receptors, delivery of cellular cargoes to the site of pathogen infection, and defence signaling (Teh & Hofius, 2014). However, the function of membrane trafficking in the immunity-related hypersensitive response (HR) remains largely unknown. The *acd11* mutant was previously shown to exhibit constitutive activation of cell death and immune responses (Malinovsky *et al.*, 2010). A genetic screen for *lazarus* (*laz*) suppressors of this autoimmune phenotype identified the TIR-NB-LRR protein LAZ5, suggesting that *acd11* cell death mimics the HR and is caused by inappropriate activation of a TIR-type immune receptor pathway in the absence of effector recognition (Palma *et al.*, 2010). It was therefore tempting to assume that other *LAZ* genes may participate in the regulation of immune receptor function and HR PCD. In this study, the characterization of the *laz4* mutant identified the retromer component VPS35B as regulator of LAZ5-dependent cell death, and revealed a critical role of retromer-mediated endomembrane trafficking and vacuolar functions in pathogen-triggered autophagy, HR, and disease resistance.

3.1.1 Identification of LAZ4 as a component of the retromer complex

By using a map-based cloning approach, *laz4* was found to carry an intron splice mutation in the *VPS35B* gene on chromosome 1, which resulted in the deletion of an entire exon from the transcript. To test whether the *laz4*-mediated effect on *acd11* cell death was indeed caused by the mutation in VPS35B, the wild-type *VPS35B* locus was introduced into Landsberg *erecta* (L*er*) *acd11 nahG laz4* plants. BTH treatment of the transgenic progenies triggered a comparable growth retardation and cell death phenotype as in the

acd11 nahG background line. Moreover, introducing independent *VPS35B* loss-of-function mutations into Col-0 *acd11 nahG* plants resulted in suppression of BTH-triggered cell death. Together, these results verified that *LAZ4* encodes VPS35B and is required for autoimmune cell death in *acd11*.

Subcellular localization and interaction studies were then performed to analyze whether VPS35B is an integral part of the retromer complex and thus acts in a comparable manner as other VPS35 homologs (VPS35A and VPS35C) in Arabidopsis (Robinson *et al.*, 2012). To this end, transgenic plants expressing a VPS35B-GFP fusion construct under control of the native promoter were generated and crossed with a series of different subcellular marker lines. VPS35B-GFP appeared in punctate structures that co-localized with the retromer component VPS29 and the MVB/PVC marker RabG3f, but not with TGN/EE or Golgi markers. Treatment with wortmannin (Wm), which enlarges the MVB/PVC due to inactivation of phosphatidylinositol 3-kinases (Figure 1) (Matsuoka *et al.*, 1995), supported the notion that VPS35B is localized to the MVB/PVC. Furthermore, VPS29 was detected in VPS35-GFP-containing immunoprecipitates, and additional bimolecular fluorescence complementation (BiFC) and yeast two-hybrid analyses confirmed the capability of VPS35B to interact with retromer core subunits. These results verified that VPS35 is part of the MVB/PVC-localized retromer complex. Consistent with our findings, other studies suggested that the core subunits of retromer are localized to MVB/PVC (Yamazaki *et al.*, 2008; Oliviusson *et al.*, 2006). However, the localization of retromer subunits to the TGN was also reported (Niemes *et al.*, 2010). The discrepancy in retromer localization might be explained by the fact the MVB/PVC matures from the TGN/EE (Singh *et al.*, 2014; Scheuring *et al.*, 2011).

3.1.2 The role of retromer in pathogen-triggered HR and autophagy

In-depth genetic analysis of LAZ4/VPS35B and its homologs suggested that VPS35-dependent trafficking contributes to autoimmunity and HR cell death triggered by TIR- and CC-NB-LRR proteins. Importantly, retromer dysfunction specifically abrogated disease resistance mediated by the TIR-NB-LRR protein RPS4 in response to the avirulent bacterial strain *Pst* DC3000 (*AvrRps4*), resembling the phenotype of the autophagy-defective mutant *atg5* (Dong & Chen, 2013). Apart from its potential contribution to endosomal sorting of immune components, retromer might regulate HR and immune responses by its function in vacuolar trafficking and integrity (Nodzynski *et al.*, 2013). Indeed, retromer deficiency caused alterations in the morphology of late endocytic and vacuolar compartments, particularly in *vps29*/*mag1-1* single and *vps35a-1 c-1* double mutants, which showed the strongest suppression of HR.

Notably, treatment with the SA analog BTH aggravated this phenotype, implying that the defects in lytic compartments may be pronounced under HR-promoting conditions.

The observed abnormalities in lytic compartments prompted us to investigate autophagy processes in retromer mutants. The *vps35a-1 c-1* and *vps29*/*mag1-1* mutants displayed comparable exaggerated senescence in response to nutrient limitation as seen in the autophagy-deficient mutant *atg2*. We then analyzed protein abundance of the cargo receptor NBR1, which is routinely used as an indicator for autophagy flux (Svenning *et al.*, 2011), and found that certain retromer mutants showed constitutively elevated NBR1 levels that remained unaffected upon additional treatment with the cysteine protease inhibitor E-64d (for further details regarding the effect of E-64d, see Figure 1). This finding revealed that retromer functions are required for the completion of autophagic degradation. Lastly, we assessed the impact of retromer dysfunction on immunity-induced autophagy and demonstrated that *vps35a-1 c-1* was clearly defective in autophagic flux triggered upon *Pst* DC3000 (*AvrRpm1*) infection. The autophagy defects observed in retromer mutants could arise from potential dysfunction of late endocytic and vacuolar compartments leading to impaired destruction of autophagic bodies and their associated cargos. Alternatively, retromer could be directly involved in the regulation of the autophagy pathway. For instance, VPS35 was identified as autophagosome-associated protein in mammalion cells using a proteomics approach (Dengjel *et al.*, 2012). In addition, a human Rab GTPase-activating protein was found to interact with ATG8/LC3 and VPS29, and proposed to function as a potential switch between autophagic and endosomal trafficking pathways (Popovic *et al.*, 2012). Finally, a recent study suggested that a VPS35 mutation, which is associated with Parkinson's disease, alters the localization and trafficking of the transmembrane autophagy protein ATG9 (Zavodszky *et al.*, 2014).

Taken together, the findings from this study link retromer function to HR and provide an important example for the participation of distinct membrane trafficking pathways during immune responses. We also demonstrated that retromer dysfunction results in autophagic defects, supporting the notion that crosstalk between endocytic and autophagic pathways exists in plants (Zhuang *et al.*, 2015). Consistent with our findings, it has been shown that retromer is required for degradation of autophagic cargos in *Drosophila* (Maruzs *et al.*, 2015). Another link between retromer and autophagy comes from the finding that retromer is important for autophagy-dependent rice infection by the fungus *Magnaporthe oryzae* (Zheng *et al.*, 2015). The complex interrelationship

between retromer-mediated trafficking and autophagy pathways in response to activated immune receptors needs to be further dissected in the future.

3.2 The role of the tonoplast-localized DUF300 protein in immunity-associated autophagy (Paper II and III)

LAZ1 encodes a DUF300 (domain of unknown function 300) transmembrane protein and shows homology to a family of conserved OST-alpha proteins implicated in post-Golgi membrane trafficking and steroid transport in animals (Olivier-Mason *et al.*, 2013; Dawson *et al.*, 2010; Best & Adams, 2009; Best *et al.*, 2008). LAZ1 was initially reported to contribute to autoimmunity in the Arabidopsis mutant *acd11* (Malinovsky *et al.*, 2010). Importantly, the *laz1* mutation suppresses autophagy-dependent HR conditioned by the NB-LRR immune receptors RPM1 and RPS4, respectively (Malinovsky *et al.*, 2010). Here we investigated the subcellular localization of LAZ1 and its closest homolog LAZ1H1 (LAZ1 HOMOLOG1) and established an important role of LAZ1 in immunity-associated autophagy.

3.2.1 Tonoplast localization of LAZ1 and LAZ1H1

In order to elucidate the functions and potential redundancy of LAZ1 and LAZ1H1, we generated *laz1 laz1h1* double loss-of-function mutants that showed severe growth inhibition. Transgenic expression of either *LAZ1-GFP* or *LAZ1H1-GFP* under control of their native promoters rescued the dwarf phenotype. This verified the functionality of the constructs, which were subsequently introduced into the respective single mutants for subcellular localization studies. Both LAZ1-GFP and LAZ1H1-GFP localized to the vacuole membrane, as demonstrated by prolonged FM4-64 staining and co-localization with the tonoplast marker VAMP711-mCherry. Additional support for their tonoplast localization was provided by the observation that *laz1 laz1h1* double mutants displayed gross malformation of the vacuoles, while other major organelles of the vacuolar trafficking pathway (i.e. TGN/EE and MVB/PVC) remained unaffected.

LAZ1 has previously been localized to the PM, the cytosol, and FM4-64-stained punctate structures upon transient expression of fluorescently tagged LAZ1 driven by cauliflower mosaic virus (CaMV) 35S promoter in Arabidopsis mesophyll protoplast (Malinovsky *et al.*, 2010). This contradictory localization pattern is most likely caused by the non-physiological conditions of high-level expression in the protoplast system and does not reflect the situation in intact plant cells. Therefore, transgenic expression of the functionally verified fusion constructs in stable Arabidopsis unequivocally

revealed that LAZ1 and LAZ1H1 reside exclusively on the vacuole membrane, thereby opening the door to an in-depth analysis of their functional roles at this particular cellular compartment.

3.2.2 LAZ1 contributes to cell death triggered by autoimmunity and hydroxyurea

In addition to its role in autoimmune cell death in *acd11*, we observed that the loss-of-function *laz1* mutation suppressed autoimmunity in the *acd5* mutant, which is disrupted in the ceramide kinase and shows ceramide accumulation and autophagy activation (Bi *et al.*, 2014; Liang *et al.*, 2003). In contrast, run-away cell death in *lsd1* was not altered by the *laz1* mutation. Interestingly, *lsd1* cell death is known to mainly require metacaspase 1, which appears to act in an autophagy-independent pathway during HR cell death (Coll *et al.*, 2014; Coll *et al.*, 2010). Previously, the autophagy-deficient mutants were reported to show resistance to hydroxyurea, a ribonucleotide reductase inhibitor commonly used in cancer therapy (Hackenberg *et al.*, 2013). Importantly, treatment with hydroxyurea leads to activation of autophagy (Chen *et al.*, 2014). We found that *laz1* was able to suppress hydroxyurea-induced cell death, partially resembling *atg* mutants. Hence, these results further supported a potential direct link of LAZ1-related functions to autophagy.

3.2.3 LAZ1 is required for the regulation of immunity-associated autophagy

To analyze whether autophagy activity is affected in *laz1*-related mutants, we examined the localization of the GFP-ATG8a fusion protein. We found constitutive accumulation of autophagosomes labeled by GFP-ATG8a in the *laz1 laz1h1* double mutant. Furthermore, autophagosomes appeared to accumulate in *laz1 laz1h1* outside of the tonoplast, as revealed by FM4-64 staining. Additionally, autophagic degradation of the adaptor protein NBR1 was particularly impaired in the double mutant, as constitutive accumulation of NBR1 was not further increased upon treatment with the cysteine protease inhibitor E64-d. Therefore, similar to certain retromer defective mutants (Paper I), autophagic degradation rather than autophagy initiation appears to be blocked in the *laz1 laz1h1* double mutant. However, a potential mechanistic difference between these mutants might be that the autophagic turnover in retromer mutants is impaired in the vacuole lumen, whereas loss of LAZ1 and LAZ1H1 may cause defects in the fusion of autophagosomes with the vacuole. The *free1*/*fyve1* mutant, which is disrupted in the late endosomal and vacuolar compartments, also displayed impaired autophagic trafficking and degradation (Gao *et al.*, 2015; Kolb *et al.*, 2015). In contrast to the double mutant, autophagy activity at basal conditions appeared to be undisturbed in *laz1* and

laz1h1 single mutants, which seems to be consistent with the normal structural appearance of their vacuoles. Future studies may address whether the *laz1* single mutant shows vacuolar dysfunction under autophagy-inducing conditions.

Both *laz1* single and *laz1 laz1h1* double mutants exhibited exaggerated senescence upon leaf detachment and prolonged darkness resembling the phenotype observed in the *atg2* mutant. Moreover, *laz1* and *laz1 laz1h1* showed a similar suppression of autophagy-dependent HR, indicating that loss-of-function of LAZ1H1 does not contribute to autophagy defects under inducing conditions. In addition, we provided genetic evidence that LAZ1 functions in autophagic processes to regulate HR cell death, as combined loss-of-function mutations in *LAZ1* and *ATG2* had no additive effect on HR suppression. In agreement with these findings, *laz1* and *laz1 laz1h1* displayed increased accumulation of NBR1 following *Pst* DC3000 (*AvrRpm1*) infection. Also, the abundance of ubiquitinated proteins, previously shown to be increased in autophagy-defective mutants during heat stress or upon *Pst* DC3000 (*AvrRpm1*) infection (Hackenberg *et al.*, 2013; Zhou *et al.*, 2013), was comparable in challenged *laz1*, *laz1 laz1h1*, and *atg2* plants.

We next examined the bacterial growth after infiltration with *Pst* DC3000 (*AvrRpm1*) and found that *laz1*-related mutants behaved similar as wild-type, supporting the previous notion that HR cell death and pathogen growth restriction are uncoupled in this incompatible host-pathogen system (Munch *et al.*, 2015; Hackenberg *et al.*, 2013; Coll *et al.*, 2010). Infection with the compatible DNA virus CaMV was assumed to be more directly affected by autophagy because autophagy-deficient mutants were shown to be substantially enhanced in viral titers compared to wild-type (Hafrén et al., 2016, personal communication and submitted). Consistent with the effect seen in *atg2* mutants, *laz1* displayed enhanced susceptibility to CaMV infection, further suggesting the induction of autophagic defects upon loss of LAZ1 function.

Collectively, these observations led to the conclusion that LAZ1 predominantly contributes to immunity-associated autophagy. The phylogenetic analysis indicates that TMEM184C/TMEM34 is the closest human homolog of LAZ1. Importantly, it has been shown that transfection of TMEM184C into anaplastic thyroid cancer cells results in inhibition of cell growth (Akaishi *et al.*, 2007), supporting the notion that LAZ1 possesses pro-death function. Our study implies the possibility that DUF300 homologues (e.g. the tumor suppressor TMEM184C) have evolutionary conserved functions in the regulation of cell death through autophagic processes, thereby shedding new light on the potential roles of DUF300 proteins in human cancer. Future studies may provide experimental validation of cross-kingdom

conservation by expression of LAZ1 in cancer cells and/or the complementation of *laz1*-related mutants with TMEM184C.

3.3 Tonoplast-localized DUF300 proteins are required for the regulation of brassinosteroid signaling (Paper II)

Brassinosteroids (BRs) control many processes during plant growth and development due to their biological activities on cell elongation, division, and differentiation (Li & Chory, 1999; Clouse & Sasse, 1998). Detailed knowledge has been accumulated on the important players of the core BR signal transduction from the receptor to downstream transcriptional regulators and thousands of target genes (Zhu *et al.*, 2013; Wang *et al.*, 2012). Recent studies have further strengthened our understanding of the biochemical and structural properties as well as the cellular dynamics of the BR receptor complex (Belkhadir & Jaillais, 2015). In this study, LAZ1 and its closest homolog LAZ1H1 were characterized as novel tonoplast-localized proteins with an important role in the regulation of BR signaling, thereby providing a mechanistic link between vacuolar functions and BR signaling.

3.3.1 The role of LAZ1 and LAZ1H1 in the BR signaling pathway

Arabidopsis mutants with vacuolar dysfunction have previously shown to be altered in endomembrane trafficking (Gu & Innes, 2012; Gendre *et al.*, 2011). We therefore analyzed *laz1 laz1h1* for changes in endocytic trafficking pathways and found that *laz1 laz1h1* displayed normal endocytosis, but slightly altered exocytosis/recycling and vacuolar trafficking of PIN proteins. In contrast to these relatively mild effects, vacuolar trafficking and degradation of the BR receptor BRI1 was substantially enhanced in the *laz1 laz1h1* double mutant. It has been suggested that the stimulation of BRI1 vacuolar targeting correlates with an activated BR signaling status (Belkhadir & Jaillais, 2015; Irani *et al.*, 2012; Russinova *et al.*, 2004). Indeed, our extensive phenotypic, biochemical, and genetic studies provided several lines of evidence that lack of LAZ1 and LAZ1H1 leads to constitutive activation of BR signaling.

Compared to wild-type, *laz1 laz1h1* showed (i) longer hypocotyls in the absence of brassinolide (BL), (ii) significant accumulation of dephosphorylated BES1 prior to BL treatment, and (iii) down-regulation of BR-repressed genes *CPD* and *DWF4*. In addition, *laz1 laz1h1* was able to revert the *bri1*-induced reduction and to aggravate the BRI1-GFP mediated increase of hypocotyl growth. Notably, *laz1 laz1h1* plants exhibited a comparable insensitivity of hypocotyl elongation to BL treatment as seen in the *bri1* mutant, which was presumed to reflect a reduction in BRI1 abundance at the plasma membrane

due to enhancement of BRI1 vacuolar degradation. It cannot be ruled out that the slightly but significantly impaired exocytosis/recycling might contribute to this phenotype. However, since PIN2 vacuolar trafficking was only mildly affected, it is unlikely that the strong influence on BRI1 subcellular distribution in *laz1 laz1h1* is caused by overall alterations in trafficking pathways of the endomembrane system. Rather, changes in PIN trafficking could be the consequence of elevated BR signaling, as PIN2 subcellular dynamics were previously shown to be altered in BR-treated wild-type as well as in the activated BR signaling mutant *act2-5* (Lanza *et al.*, 2012).

Importantly, *vps35a-1 c-1*, *pat2-2*, and *vti11* mutants that show similar alterations in vacuole morphology as *laz1 laz1h1* (Munch *et al.*, 2015; Feraru *et al.*, 2010; Yano *et al.*, 2003) exhibited a normal BR response, supporting the notion that the constitutive BR response phenotype in *laz1 laz1h1* cannot be attributed to morphological alteration in vacuoles *per se*. Therefore, DUF300 proteins on the tonoplast seem to play distinct roles in vacuole-mediated regulation of BR signaling.

3.3.2 LAZ1 and LAZ1H1 modulate BAK1 homeostasis

The BRI1 co-receptor BAK1 was previously shown to localize not only to the plasma membrane and endomembrane compartments, but also to the tonoplast (Bucherl *et al.*, 2013). However, the role of intracellular pools of BAK1 in the regulation of BRI1 receptor distribution and BR signaling has not been addressed experimentally. In fact, based on recent concerns on the functionality of epitope and fluorescent protein fusions to the C-terminus of BAK1, doubts remained whether the localization pattern of the BAK1-mCherry fusion protein represents the situation of the endogenous counterpart. We therefore used transgenic lines that expressed *pBAK1:BAK1-GFP* in Col-0 wild-type and the complemented *bak1-4* mutant background (Albrecht *et al.*, 2012; Ntoukakis *et al.*, 2011), and observed similar subcellular localization pattern including tonoplast association. We subsequently introduced BAK1-GFP into the *laz1 laz1h1* background to examine the impact of loss of LAZ1 and LAZ1H1 on BAK1 homeostasis. Intriguingly, tonoplast accumulation of BAK1-GFP was increased in *laz1 laz1h1*, whereas the levels of BAK1-GFP at the plasma membrane remained essentially unchanged. In addition, impaired vacuolar acidification and proteolytic capacity under dark conditions also resulted in an augmented tonoplast pool of BAK1-GFP in wild-type, suggesting that altered BAK1-GFP localization in *laz1 laz1h1* is likely due to impaired vacuolar functions leading to decreased BAK1 turnover (Figure 5).

Increased levels of BAK1 are known to affect BRI1 trafficking and BR signaling (Russinova *et al.*, 2004; Li *et al.*, 2002; Nam & Li, 2002), and thus

could explain the BR-related phenotypes in *laz1 laz1h1*. It has been shown that tonoplast-associated StSERK3, the BAK1 ortholog in potato is recruited to late endosomes and diverted to the extrahaustorial membrane upon pathogen infection (Bozkurt *et al.*, 2015). Responding to growth-related stimuli, we assume that tonoplast-resident BAK1 can utilize the same trafficking strategy to reach the plasma membrane, resulting in the promotion of BR signaling and hence the removal of BRI1 from the plasma membrane in a manner that depends on BRI1-BAK1 heterodimerization (Figure 5) (Bucherl *et al.*, 2013; Russinova *et al.*, 2004). However, it is important to note that the trafficking routes of BAK1 and BRI1 seem to be substantially different, as BAK1 is largely absent form BFA bodies (Bucherl *et al.*, 2013) and BRI1 does not localize to the tonoplast. Therefore, future studies may address how BAK1 traffics in the cell, and how manipulation of the distinct subcellular pools of BAK1 could affect BR signaling and other BAK1-regulated processes (e.g. immunity).

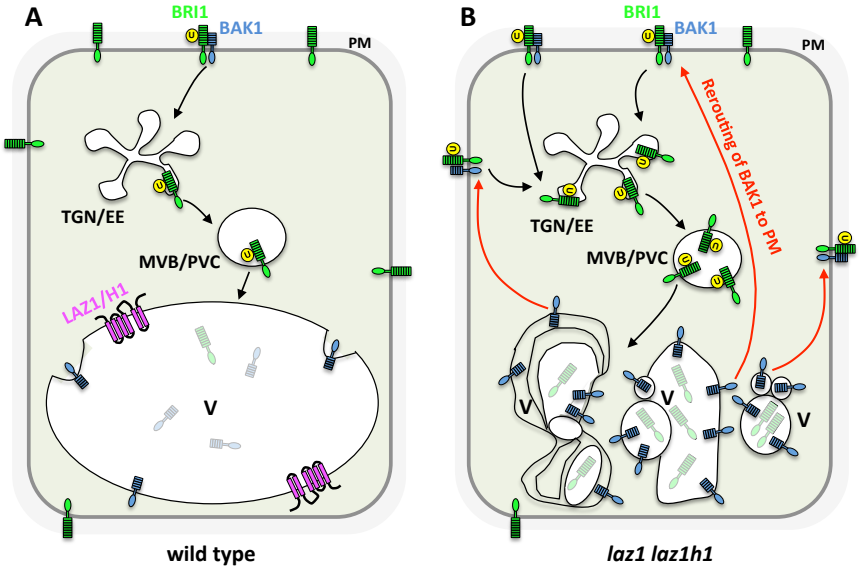

Figure 5. Schematic model for vacuole-mediated control of BR signaling. (A) In wild-type plants, LAZ1 and LAZ1H1 participate in vacuole membrane integrity, which is required for the turnover of tonoplast-localized BAK1. (B) In *laz1 laz1h1* double mutants, the levels of tonoplast-localized BAK1 are increased due to impaired vacuolar functions leading to decreased BAK1 turnover. Excessive tonoplast-associated BAK1 could be rerouted to the plasma membrane, which results in activated BR signaling and removal of BRI1 from the plasma membrane for vacuolar degradation. Ubiquitination of BRI1 has recently been shown to regulate its internalization and vacuolar transport (Martins *et al.*, 2015).

It is known that BR signaling is activated in darkness to promote plant growth (Kim *et al.*, 2014). Apart from the current model, which is mainly based on the interplay between PIF4 and HY5 with BZR1 (Li & He, 2016; Kim *et al.*, 2014; Oh *et al.*, 2012), accumulation of BAK1 in darkness might represent an alternative mechanism to explain this phenomenon.

3.4 Genetic enhancement of autophagic flux improves the fitness of plants (Paper IV)

Autophagy is an intracellular trafficking and vacuolar degradation system with implications for organismal development, health and disease. A growing body of evidence suggests that the artificial manipulation of autophagy seems to be a potent tool in a wide spectrum of biomedical and clinical applications. The development of crop varieties with improved stress tolerance is one of the main goals for plant breeding. In this study, we present a proof of concept that genetic stimulation of the autophagic flux in Arabidopsis improves various agronomically important traits without apparent fitness costs.

3.4.1 Constitutive overexpression of *ATG5* or *ATG7* stimulates autophagy in Arabidopsis

The role of autophagy in plant biology has been investigated mainly based on characterization of autophagy-deficient mutants in Arabidopsis (Liu & Bassham, 2012). To distinguish between direct and pleiotropic effects caused by inactivation of autophagy, an alternative gain-of-function approach leading to elevated autophagy levels would be suitable to address in more detail the functions of autophagy in plants. It has been demonstrated that autophagy is activated in transgenic Arabidopsis lines overexpressing a constitutively active form of RabG3b, but other unknown pathways appear to be activated in this line as well (Kwon *et al.*, 2013). In this study, several independent homozygous transgenic lines constitutively overexpressing *ATG5* or *ATG7* were generated. To examine the impact of *ATG* overexpression on autophagy initiation and flux, ATG8 lipidation and vacuolar degradation of the autophagy adaptor protein NBR1 were determined (Bassham, 2015). These assays unequivocally revealed that *ATG5* and *ATG7* overexpression resulted in a constitutive enhancement of autophagy levels and flux in these lines. Thus, *ATG*-overexpressing lines may represent a valuable genetic resource to further study the developmental and physiological roles of autophagy in plants.

Transient overexpression of *ATG3* in *Nicotiana benthamiana* leaves via agroinfiltration has recently been demonstrated to stimulate autophagy (Han *et al.*, 2015). This result together with our findings (i.e. transgenic lines stably

overexpressing *ATG5* or *ATG7*), suggests that transcriptional manipulation of genes encoding components of the ATG12-ATG5 and ATG8-phosphatidylethanolamine (ATG8-PE) conjugation systems is sufficient to enhance autophagy levels in plants.

3.4.2 Enhanced autophagy leads to a stimulating effect on plant production and stress tolerance

Autophagy-defective mutants are known to exhibit an accelerated senescence phenotype (Doelling *et al.*, 2002; Hanaoka *et al.*, 2002). In contrast, enhanced autophagy was found to suppress aging and sustain flowering. Furthermore, overexpression of *ATG* genes promoted vegetative biomass production and seed yield, while inactivation of autophagy caused the opposite effect (Bassham *et al.*, 2006). Because the weight of individual seed remained unaffected in the overexpressor lines, the elevated seed set might be caused by enhanced fecundity, which was presumed to reflect the prolonged duration of the flowering period.

Autophagy was previously reported to positively regulate plant disease resistance to necrotrophic fungal pathogens due to the observed increased susceptibility in *atg* mutants (Lai *et al.*, 2011; Lenz *et al.*, 2011). Consistent with this assumption, we observed that *ATG5* or *ATG7* overexpression resulted in enhanced resistance to the necrotrophic fungus *Alternaria brassicicola* and *Botrytis cinerea*. Thus, these results provided evidence that autophagy might be directly involved in plant defence to necrotrophic pathogens. Such contribution could derive from the suppression of pathogen-induced cell death via autophagic clearance of damaged and toxic cellular contents. Alternatively, autophagy is known to positively regulate the jasmonic acid (JA) signaling pathway (Lai *et al.*, 2011), which is required for resistance against necrotrophs. Since oxidative stress is thought to contribute to necrotrophic pathogenicity (Choquer *et al.*, 2007; AbuQamar *et al.*, 2006), it is likely that *ATG*-overexpressing plants could show enhanced resistance to oxidative stress. Indeed, the overexpressing lines proved to be more resistant to methyl viologen (MV)-induced oxidative stress compared with the wild-type control, whereas *atg* mutants were more susceptible.

Autophagy has been proposed to contribute to the initiation of immune receptor-conditioned hypersensitive response (HR) upon recognition of certain avirulent strains of the biotrophic oomycete *Hyaloperonospora arabidopsidis* and hemibiotrophic bacterium *P. syringae* (Minina *et al.*, 2014; Hofius *et al.*, 2009). Intriguingly, *ATG*-overexpressing plants showed elevated ion leakage upon *Pst* DC3000 (*AvrRpm1*) infection, suggesting that enhanced autophagic flux activates HR cell death. In agreement with the notion that host HR and

pathogen growth restriction are uncoupled (Munch *et al.*, 2015; Hackenberg *et al.*, 2013; Coll *et al.*, 2010), bacterial growth of the avirulent strain was not altered in overexpressor lines or *atg* mutants. Furthermore, we spray-inoculated *ATG5* overexpressing lines and *atg5* mutants with virulent *Pst* DC3000. We found that bacterial growth was increased in *ATG5* overexpressing lines, whereas *atg5* mutants appeared to be slightly, yet not significantly more resistant compared to the wild-type (Figure 6). One tempting explanation for this outcome is that autophagy may directly down-regulate the levels of the defence hormone salicylic acid, e.g. by targeting SA-related cargo to the vacuole for degradation (Kulich & Zarsky, 2014).

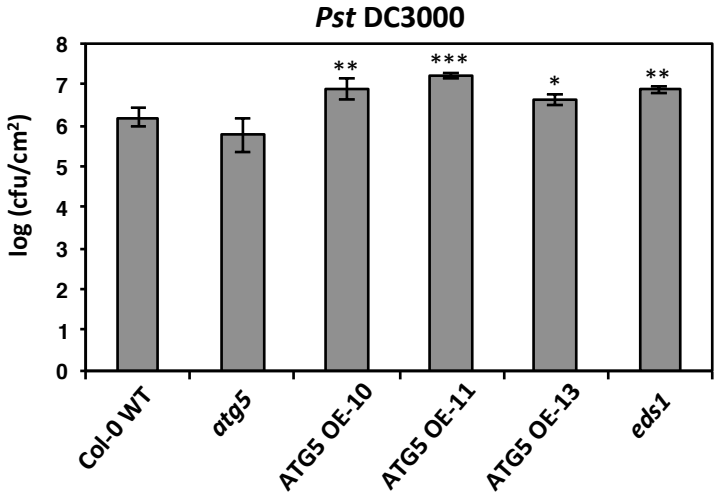

Figure 6. Elevated levels of autophagy results in increased susceptibility to infection with virulent bacteria. Growth of *Pst* DC3000 in 6-week-old Col-0 wild-type, *atg5* mutants, ATG5 overexpressor lines (OE), and *eds1* mutants 3 days after spray inoculation at OD600 = 0.1. Log-transformed values are means ± standard deviation (SD) with n = 4. cfu, colony-forming units. Asterisks indicate statistical significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) determined by Student's *t* test (compared with Col-0 wild-type).

Consistent with our findings in plants, overexpression of *ATG5* in mice was shown to induce autophagy (Pyo *et al.*, 2013), which resulted in increased longevity, leanness, improved insulin sensitivity, enhanced motor function and tolerance to oxidative stress (Pyo *et al.*, 2013). Therefore, our data (Figure 7) taken together with this previous report provide strong evidence for a cross-kingdom conservation of a stimulating effect of enhanced autophagy activity on organismal fitness. The improvement of agricultural crops through genetic stimulation of autophagy would be an important goal in the future. Apart from

overexpression of *ATG* genes, other methods could also be used to enhance autophagy, including (i) the application of chemical drugs stimulating *ATG* gene transcription, (ii) the modification of epigenetic regulators of *ATG* genes, and (iii) the genetic disruption of negative regulator of autophagy via CRISPR/Cas-mediated genome editing.

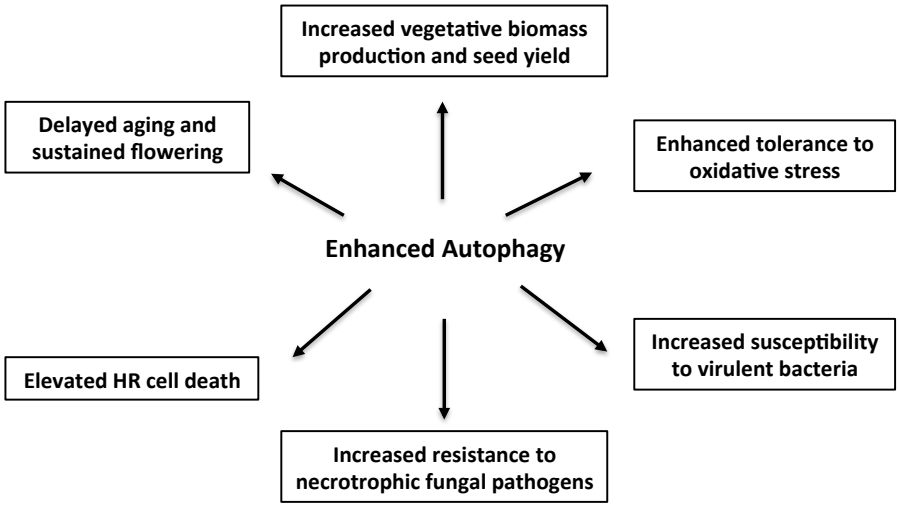

Figure 7. Schematic overview of the consequences of enhanced autophagy on plant growth and stress tolerance. Details have been described in the section 3.4.

4 Conclusions

The key findings from the work presented in this thesis are:

LAZ4 encodes one of three Arabidopsis VPS35 proteins, which are core components of the retromer complex. *VPS35* genes contribute to certain forms of autoimmunity, pathogen-triggered hypersensitive response (HR), and disease resistance conditioned by NB-LRR immune receptors. Retromer-dependent processes maintain the integrity of late endocytic/lytic compartments and are critical for vacuole-mediated autophagic degradation under basal and immunity-associated conditions.

The DUF300 proteins LAZ1 and its closest homolog LAZ1H1 localize to the tonoplast. Combined loss of LAZ1 and LAZ1H1 results in dwarfism, altered vacuole morphology, and constitutive activation of brassinosteroid (BR) signaling. Tonoplast accumulation of the BRI1 co-receptor BAK1 is substantially increased in the *laz1 laz1h1* mutant, which we propose is causally linked to accelerated vacuolar trafficking and degradation of BRI1. The resulting depletion of plasma membrane-associated BRI1 rendered *laz1 laz1h1* plants insensitive to BR treatment. Unrelated vacuole mutants show normal BR responses, indicating that LAZ1 and LAZH1 play distinct functions in the vacuole-mediated regulation of BR signaling.

The *laz1 lazh1* double mutant is impaired in autophagy processes under basal conditions. A comparable autophagy defect is detected in *laz1* but not in *laz1h1* single mutants upon nutrient starvation and pathogen-triggered HR, indicating that LAZ1 is the main contributor to autophagy function. Consistent with this, the absence of LAZ1 is sufficient to compromise different forms of

immunity-related cell death as well as autophagy-dependent antiviral disease resistance.

Enhancement of autophagy levels and flux in Arabidopsis can be achieved by constitutive overexpression of the autophagy-related genes *ATG5* or *ATG7*. The resulting promotion of pathogen-triggered HR as well as improved resistance to oxidative stress and infection with necrotrophic fungal pathogens are opposite to the effects observed in autophagy-deficient mutant, thereby verifying the positive role of autophagy in these processes. Furthermore, enhanced autophagy results in the promotion of vegetative growth and seed production, suggesting that genetic stimulation of the autophagy pathway is a promising strategy to improve both productivity and stress tolerance in plants.

5 Future perspectives

The results of this thesis hold great importance to improve our current understanding of vacuole-mediated control of plant growth and immunity and encourage further exploration.

To identify the molecular pathways relevant for the functions of LAZ1/LAZ1H1, we initiated a genetic screen for suppressors of the severe dwarf phenotype of *laz1 laz1h1* in M2 populations of ethyl methanesulfonate (EMS)-mutagenized seeds. We have so far identified more than 30 revertants with improved rosette and inflorescence growth compared to *laz1 laz1h1*, and found two strong suppressors that segregate according to a single recessive mutation in the F2 population derived from a backcross to the parental line. Identification and characterization of these suppressor genes will greatly facilitate our understanding of LAZ1/LAZ1H1 functions in relation to vacuole homeostasis, BR signaling, autophagy and immune responses.

The cytosolic C-terminus of LAZ1 has previously used as bait in a yeast two-hybrid screen, and identified the 14-3-3 protein GRF6 (also known as 14-3-3λ) as the only potential LAZ1 interactor (F. Malinovsky, PhD thesis, Copenhagen University). GRF6 has been shown to interact with several proteins implicated in the BR signaling pathway (e.g. SERK1, BZR1, BES1) as well as in effector-triggered immunity (e.g. RPW8 R protein) (Chevalier *et al.*, 2009; Yang *et al.*, 2009). We have verified the interaction of LAZ1 and LAZ1H1 on the tonoplast membrane by bimolecular fluorescence complementation (BiFC) upon transient expression in *N. benthamiana*. Future studies may address whether LAZ1 acts in BR signaling and/or immunity-related cell death through its interaction with GRF6.

Although the biochemical function of LAZ1 is still unknown, a clue may come from its mammalian homolog Ostα, which has been implicated in bile acid and steroid transport (Dawson *et al.*, 2010). Bile acids are not found in plants, but the primary bile acid, cholic acid (CA), was reported to induce

hypersensitive cell death and defence responses in rice (Koga *et al.*, 2006). CA is structurally similar to the insect steroid ecdysone, which triggers developmental autophagic cell death in *Drosophila* (Rusten *et al.*, 2004), and to brassinosteroids involved in plant immunity and autophagic PCD associated with tracheary element differentiation (Lozano-Duran & Zipfel, 2015; Kwon *et al.*, 2010). It will be very interesting to investigate whether plant steroid-related compounds in general possess death- and autophagy-promoting functions and could be transported by DUF300 proteins.

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Acknowledgements

It was really a great pleasure to complete my PhD education here in the Department of Plant Biology at SLU in Uppsala. I would like to express my deep and warm gratitude to all the people who helped and encouraged me during this period.

First of all, I wish to acknowledge my main supervisor Professor Daniel Hofius for his continuous support, great patience, excellent scientific guidance and supervision. In particular, I am very grateful for the freedom he has allowed to develop my own ideas, thereby letting me become a motivated and independent researcher.

I also wish to acknowledge my co-supervisors, Lars Hennig and Peter Bozhkov, for their support, help and collaborations throughout the period of my PhD study.

I would like to thank Eva Sundberg, Mattias Thelander and Sarosh Bejai for their valuable input and suggestions during the PhD evaluation process.

Thanks to all the present and past members of our group, Anders Hafrén, Ooi-kock Teh, Ramesh Vetukuri, and Suayib Üstün. We have spent a lot of time together and I would like to thank you all for your nice company, advice, help, and friendship over the years.

Thanks a lot, Panos and Alyona, for your help especially at the initial stage of my PhD and your suggestions on my projects. Thank you, Christina Dixelius, Folke and Sun for your help and encouragement. Thanks to Reza, Malin, Tianqing, Anna, Sultana, Ming, Rita and Hanneke for the nice time during teaching. Thanks to Emilio, Pascal, Hua, Guifeng, Rocky, Iva, Shujing, Jun,

Irene, Shirin, Christina Roberts, Kai, Mingliang, Clément, Cecilia, Jordi, Emma: you all helped me in various ways.

Thanks to Urban and Per for the great assistance to keep my plants happy. I would like to thank Björn for the computer support. I also wish to thank our administrative staff, Mona, Anneli, Lotta, Monica and particularly Qing for the generous help during these years.

I pay special thanks to Stéphanie Robert for hosting my study visit at the Umeå Plant Science Centre for three times. I am truly grateful to the members of the Robert lab: Thomas, Qian, Siamsa, Mateusz, Adeline.

I also want to express my sincere gratitude and appreciation to other friends that I met in Sweden. Thanks for making my life here colorful.

Last but not least, I would like to give my distinctive thanks to my parents and grandparents. Thank you for your enduring love, support, tolerance and encouragement.