PhD thesis
David Munch

Autophagy and retromer components in plant innate immunity

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Department of Biology
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Preface & acknowledgements

This thesis concludes my PhD at the Department of Biology, University of Copenhagen, 2014. The work presented here has contributed to one review, two submitted manuscripts and one accepted manuscript, which are attached at the end of this thesis. All papers involve plant immunity and associated cell death.

I would like to thank my supervisors John Mundy and Morten Petersen for giving me the pleasure of working with the presented topics. Its been a roller coaster ride with both ups and downs, but I made it past the goal line with your help.

A big thank you goes to the Nordic Autophagy Network (Nordforsk), for supporting me financially during my visit in Oslo, Norway (And for presenting me with very fine nordic cuisine at the Oslo meeting!).

While most of our conversations were not face-to-face, I have to give an enormous thank you to Anne Simonsen for allowing me to stay in her lab, and another giant thank you to Maria Torgersen for making sure I had a blast while being in Oslo. My stay gave me the opportunity to get to know some of the nicest people I have ever met! Alf, Maria, Helene, Pauline, Serhiy, Kristiane, Petter and Gunnveig, I sincerely miss you guys!

Thanks to Søren Skov for letting me do research in his lab, and thanks to Lars Andresen for making sure that I did not mess up too much during my visits.

And I shouldn’t forget the people in the PMB lab - Thank you guys for giving me several lovely years! I hope you will all survive without hearing more about LAZ4, and I will make sure to bring one last cake to the office! Last, thank you Louise, for being there whenever I needed you.

-David
Abstract

Innate immunity depends on the recognition of pathogens and subsequent regulation of complex interactions that ultimately leads to production of compounds to deter microbial innovation. This thesis presents different aspects of immunity-associated cell death with focus on autophagy in the plant *Arabidopsis* and human cells.

ACCELERATED-CELL-DEATH11 (ACD11) is an *Arabidopsis* mutant that shows spontaneous and lethal cell death dependent on activation of the resistance protein LAZ5, a cytoplasmic receptor of the TIR-NB-LRR type. In a screen for suppressors of *acd11*-associated cell death, *laz4*, a component of the retromer complex, was discovered as a relatively weak suppressor. Here I show redundancy between the three VPS35 homologs present in *Arabidopsis* in regulation of immunity-associated cell death, with a focus on the catabolic pathway autophagy. In addition a role for ACD11 in sphingolipid metabolism and its role in plant innate immunity will be presented.

A homolog of ACD11 in humans is FOUR-PHOSPHATE ADAPTOR PROTEIN2 (FAPP2) and it has also been shown to be involved in cell death regulation in human Jurkat T cells. The data presented here show that FAPP2 does not appear to be involved in cell death regulation in human HeLa cells, contrasting previous observations.

Autophagy is a catabolic pathway involved in both pro-life and pro-death regulation of stress and cell death. The pathway has previously been shown to be controlled through NPR1, a central regulator for the phyto-hormone salicylic acid. Here, I present data that make it clear that NPR1 does not directly regulate autophagy, but instead control stress responses that indirectly activate autophagy. The observations presented will also clarify why autophagy has been described as being both a pro-death and pro-life pathway under similar conditions.
Abstrakt

Innat immunitet afhænger af genkendelsen af patogener og den efterfølgende regulation af komplekse interaktioner som i sidste ende leder til produktionen af kemiske forbindelser for at stoppe mikrober. Denne afhandling præsenterer forskellige aspekter af immunitet-associeret celledød, med fokus på autophagy i planten *Arabidopsis* og humane celler.


En homolog af ACD11 i mennesker er FOUR-PHOSPHATE ADAPTOR PROTEIN2 (FAPP2) og den har også vist sig at være involveret i celledødsregulering i humane Jurkat T celler. Dataet præsenteret her viser at FAPP2 ikke umiddelbart er involveret i celledødsregulering i humane HeLa celler, hvilket er modstridende med tidligere observationer.

Autophagy er en katabolsk pathway involveret i både pro-liv og pro-død regularering af stress og celledød. Pathwayen er tidligere blevet vist til at være kontrolleret gennem NPR1, en central regulator af plantehormonet salicylsyre. Her præsenterer jeg data som gør det klart at NPR1 ikke direkte regulerer autophagy, men istedet kontrollerer stress responses som indirekte aktiverer autophagy. Observationerne vist her vil også gøre det klar hvorfor autophagy har været beskrevet som værende både en pro-død og pro-liv pathway under lignende tilstande.
List of publications and manuscripts

• Retromer controls immunity associated cell death in Arabidopsis.
  David Munch, Qinsong Liu, Frederikke Gros Malinovsky, Ramesh R. Vetukuri, Peter Brodersen, Ikuko Hara-Nishimura, Jeffery L. Dangl, Morten Petersen, John Mundy, and Daniel Hofius. Awaiting final figures for re-submission to Plant Cell.

• Autophagy deficiency leads to accumulation of ubiquitinated proteins, ER stress and cell death in Arabidopsis.

• Arabidopsis accelerated-cell-death11, ACD11, is a ceramide-1-phosphate transfer protein and intermediary regulator of phytoceramide levels.

• Role of autophagy in disease resistance and hypersensitive response-associated cell death.

All manuscripts and publications are included at the end of the thesis.

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>At - <em>Arabidopsis thaliana</em></td>
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<tr>
<td>ACD</td>
<td>Accelerated cell death</td>
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<tr>
<td>ATG</td>
<td>autophagy-related gene</td>
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<tr>
<td>Aza</td>
<td>Azelaic acid</td>
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<tr>
<td>BAK1 - BRI1-ASSOCIATED RECEPTOR KINASE1</td>
<td></td>
</tr>
<tr>
<td>BIK1 - BOTRYTIS-INDUCED KINASE1</td>
<td></td>
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<tr>
<td>BKK1 - BAK1-LIKE1</td>
<td></td>
</tr>
<tr>
<td>BTH</td>
<td>Benzothiadiazole</td>
</tr>
<tr>
<td>C1P</td>
<td>Ceramide-1-phosphate</td>
</tr>
<tr>
<td>CC</td>
<td>Coiled-coiled</td>
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<tr>
<td>C. elegans - <em>Caenorhabditis elegans</em></td>
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</tr>
<tr>
<td>CERK1 - CHITIN ELICITOR RECEPTOR KINASE1</td>
<td></td>
</tr>
<tr>
<td>CNGC - CYCLIC NUCLEOTIDE-GATED CHANNEL</td>
<td></td>
</tr>
<tr>
<td>Col - Columbia</td>
<td></td>
</tr>
<tr>
<td>DA - Abietane diterpenoid dehydroabietinal</td>
<td></td>
</tr>
<tr>
<td>DAMP - Damage-associated molecular pattern</td>
<td></td>
</tr>
<tr>
<td>DND - DEFENSE, NO DEATH</td>
<td></td>
</tr>
<tr>
<td>EDS1 - ENHANCED DISEASE SUSCEPTIBILITY1</td>
<td></td>
</tr>
<tr>
<td>EDS5 - SA INDUCTION-DEFICIENT1</td>
<td></td>
</tr>
<tr>
<td>EFR - EF-TU RECEPTOR</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>ET</td>
<td>Ethylene</td>
</tr>
<tr>
<td>ETI</td>
<td>effector-triggered immunity</td>
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<tr>
<td>FAPP2 - FOUR-PHOSPHATE-ADAPTOR PROTEIN2</td>
<td></td>
</tr>
<tr>
<td>FB1 - Fumonisin B1</td>
<td></td>
</tr>
<tr>
<td>FLS2 - FLAGELLIN-SENSITIVE2</td>
<td></td>
</tr>
<tr>
<td>G3P</td>
<td>Glycerol-3-phosphate</td>
</tr>
<tr>
<td>GLTP - GLYCOLIPID TRANSFER PROTEIN</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitivity response</td>
</tr>
<tr>
<td>HSP90</td>
<td>HEAT-SHOCK PROTEIN90</td>
</tr>
<tr>
<td>INA</td>
<td>2,6- dichloroisonicotinic acid</td>
</tr>
<tr>
<td>IL-1</td>
<td>INTERLEUKIN-1</td>
</tr>
<tr>
<td>IRE1 - INOSITOL-REQUIRING ENZYME1</td>
<td></td>
</tr>
<tr>
<td>JA - Jasmonate</td>
<td></td>
</tr>
<tr>
<td>LAZ - LAZARUS</td>
<td></td>
</tr>
<tr>
<td>LCB</td>
<td>Long chain base</td>
</tr>
<tr>
<td>Ler - Landsberg</td>
<td></td>
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<tr>
<td>LMM</td>
<td>Lesion mimic mutant</td>
</tr>
<tr>
<td>LSD</td>
<td>Lesion simulating disease</td>
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<tr>
<td>MAMP</td>
<td>Microbe-associated molecular pattern</td>
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<tr>
<td>MOO</td>
<td>Mitochondrial outer membrane</td>
</tr>
<tr>
<td>MPK</td>
<td>MITOGEN-ACTIVATED PROTEIN KINASE4</td>
</tr>
<tr>
<td>MTI</td>
<td>MAMP-triggered immunity</td>
</tr>
<tr>
<td>NDR1</td>
<td>NONRACE-SPECIFIC DISEASE RESISTANCE1</td>
</tr>
<tr>
<td>NPR</td>
<td>NON-EXPRESSOR OF PR-GENES</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAD4</td>
<td>PHYTOALEXIN DEFICIENT4</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PH</td>
<td>Plekstrin homology</td>
</tr>
<tr>
<td>PI3P</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PR</td>
<td>Pathogenesis-related</td>
</tr>
<tr>
<td>PRA1 - PRENYLATED RAB ACCEPTOR</td>
<td></td>
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<tr>
<td>PRR</td>
<td>Pattern recognizing receptor</td>
</tr>
<tr>
<td>PdIns(#P)</td>
<td>- Phosphatidylinositol-#-phosphate</td>
</tr>
<tr>
<td>R</td>
<td>R gene / protein - Resistance gene / protein</td>
</tr>
<tr>
<td>RIN4 - RPM1-INTERACTING PROTEIN4</td>
<td></td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPM1</td>
<td>RESISTANCE TO <em>PSEUDOMONAS SYRINGAE</em></td>
</tr>
<tr>
<td>PV</td>
<td>MACULICOLA1</td>
</tr>
<tr>
<td>RPS2</td>
<td>RESISTANCE TO <em>PSEUDOMONAS SYRINGAE2</em></td>
</tr>
<tr>
<td>SAG101</td>
<td>SENESCENCE-ASSOCIATED GENE101</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>Systemic acquired resistance</td>
</tr>
<tr>
<td>SID2</td>
<td>ISOCORISMATE SYNTHASE1</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SNX</td>
<td>Sorting nexin</td>
</tr>
<tr>
<td>SPT</td>
<td>SERINE PALMITOYLTRANSFERASE</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll and Interleukin-1 Receptor homology</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TM</td>
<td>Tunicamycin</td>
</tr>
<tr>
<td>TOR</td>
<td>TARGET OF RAPAMYCIN</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VAP</td>
<td>VESICLE-ASSOCIATED MEMBRANE PROTEIN-ASSOCIATED PROTEIN</td>
</tr>
<tr>
<td>VPE</td>
<td>Vacuolar processing enzyme</td>
</tr>
<tr>
<td>VPS</td>
<td>VACUOLAR PROTEIN SORTING</td>
</tr>
<tr>
<td>VSR</td>
<td>Vacuolar sorting receptor</td>
</tr>
<tr>
<td>VSV-G</td>
<td>VIRUS G PROTEIN</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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1. Introduction

Plants live in complex ecosystems where they are continuously exposed to various microorganisms such as bacteria, fungi and viruses. In the majority of these plant-microbe interactions the plant is unaffected, as the potentially pathogenic microbes are not adapted to the host species. However, for a subset of microbial species, the defensive capabilities of specific plant species have been overcome. They have evolved to evade or disable the defensive strategies employed against them, succeeding in colonization and subverting host tissues to provide vital nutrients. These adaptations are the result of an evolutionary arms race for compatibility, which has led to the development of both passive and inducible defense mechanisms employed by plants to maintain a status quo (Jones & Dangl, 2006).

The following section will give an introduction to current views on plant immunity and associated cell death, with emphasis on aspects of plant-pathogen interactions that are relevant to the work of this thesis. The role of sphingolipid metabolism in innate immunity will be mentioned, as well as the parallels that can be drawn between plant and mammalian innate immunity, when relevant.

1.1 Plant immunity

Phytopathogens have different lifestyles with accompanying penetration mechanisms that are specifically suited to infect only a limited number of plant species. As a consequence, plants have to employ a complex array of defenses to maintain immunity (Fan & Doerner, 2012). As plants lack an adaptive immune system, or a circular vascular system with mobile immunity-associated cells, plant cells rely entirely on an innate immune system.

Preformed passive defensive mechanisms form the first set of obstacles that microorganisms have to overcome to establish infection (Thordal-Christensen, 2003). Physical structures such as a cuticular wax barrier on leaves and closure of stomata provide the first hindrance. In addition, plants constitutively produce chemicals such as
antimicrobial compounds and toxic secondary metabolites that are secreted and maintained in tissues. Microbes that do not succumb to this type of defense and succeed in invading, either through wounds or through open stomata, are then faced with the cell walls which are designed to both shape and stabilize the plant cell and to act as a physical encumbrance to pathogens (Mysore & Ryu, 2004; Senthil-Kumar & Mysore, 2013). While these passive measures are sufficient to ward off most intruders, plants also deploy inducible defenses that respond to the recognition of pathogens in the form of a complex bi-layered defense system (Jones & Dangl, 2006). Some microbes have adapted to this and actively target plant cell signaling nodes to suppress the capability to induce defensive responses. In some cases this has led to the active recruitment or manipulation of specific subsets of the defense response to further the virulence of the pathogens (Jones & Dangl, 2006; Lai & Mengiste, 2013).

1.2 The first surveillance system

The first tier of the inducible defense system is comprised of an array of transmembrane receptors in the plasma membrane of plant cells which recognize evolutionary conserved microbe-associated molecular patterns (MAMPs) and trigger a response (Antolín-Llovera et al., 2012; Dubery et al., 2012; Zhang & Zhou, 2010). In addition, plants are capable of sensing damage-associated molecular patterns (DAMPs) that are released during tissue damage, in an autocrine fashion, emphasizing an ability for the capacity to distinguish between self and non-self (Albert, 2013).

The genome of Arabidopsis thaliana (At) has more than 650 receptor-like kinases and receptor-like proteins (Fritz-Laylin et al., 2005; Shiu & Bleecker, 2003), providing a wide repertoire of ways to detect extracellular pathogens. Through evolution these pattern recognizing receptors (PRRs) have been selected to detect structural components indispensable for microbe survival, which in some cases are conserved across whole classes of microbes (Zipfel, 2008). This first line of inducible defenses is referred to as MAMP-triggered immunity (MTI), and several examples of MAMPs and their corresponding PRRs have been characterized (Figure 1.1; Muthamilarasan & Prasad, 2013).
An example is the FLAGELLIN-SENSITIVE2 (FLS2) receptor-like kinase that recognizes a conserved part of the flagellum of certain bacterial species (Gomez-Gomez & Boller, 2000). Upon binding of the flagellin ligand, FLS2 forms a complex with BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) and BOTRYTIS-INDUCED KINASE1 (BIK1) which initiates a series of transphosphorylation events and activation of defense responses and subsequent ubiquitination leading to internalization of the FLS2 receptor (Chinchilla et al., 2007; Heese et al., 2007; D. Lu et al., 2010; Robatzek, Chinchilla, & Boller, 2006; Schulze et al., 2010; Zipfel et al., 2004)). Translocation from the plasma membrane to the endosomal network does not stop immediate defense signaling, but ultimately leads to degradation of the receptor in a proteasomal-dependent manner (Beck et al., 2012; D. Lu et al., 2011; Robatzek et al., 2006). Several other PRRs with roles in MTI have subsequently been identified (Muthamilarasan & Prasad, 2013; Newman et al., 2013): the CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) that perceives chitin from fungal cell walls (Miya et al., 2007), EF-TU RECEPTOR (EFR) that recognizes part of the bacterial elongation factor EF-Tu (Kunze et al., 2004; Zipfel et al., 2006), and Eix1 and Eix2 in tomato that detect fungal xylanase (Ron & Avni, 2004).

**Figure 1.1:** Schematic example of the first layer of surveillance. MAMPs are detected by PPRs which active defense responses, ultimately leading to the expression of defense-specific gene expression.
To overcome the plant armory, pathogens have evolved effectors that interfere with MTI to increase microbial virulence during infection. These virulence effectors are secreted into the apoplast or directly into the cytoplasm of host cells (Giraldo & Valent, 2013; Lindeberg et al., 2012). While gram-negative bacteria use a membrane-penetrating needle as a secretion system (Büttner & Bonas, 2006; Deane et al., 2010; Holland, 2010), fungi seem to deliver effectors using the appressoria, thus exploiting the close proximity of fungal-host membranes (Giraldo & Valent, 2013). In the case of the hemi-biotrophic bacteria Pseudomonas syringae pv. tomato (Pst), at least 29 effectors are secreted into the cytoplasm, while the obligate biotroph Hyaaloperonospora arabidopsidis (Hpa) is believed to secrete more than 130 effectors, exemplifying that this arms race has been evolving for some time (Chang et al., 2005). The secretion systems are thus vital for pathogenicity, as inhibition of these structures greatly lowers infection due to non-suppressed host defense (Deng et al., 1998). After injection, these virulence effectors target signaling nodes at all levels to disrupt defense regulation, and a large repertoire of effectors from different pathogens have now been characterized (Giraldo & Valent, 2013; Lindeberg et al., 2012). Examples of effectors from Pseudomonas are avrPto that directly interacts with and suppresses FLS2 and EFR by interfering with transphosphorylation (Shan et al., 2008; Xiang et al., 2011), avrB that activates MITOGEN-ACTIVATED PROTEIN KINASE4 (MPK4) in a HEAT-SHOCK PROTEIN90-dependent (HSP90) manner to perturb hormone signaling (Cui et al., 2010; Grant et al., 1995), or HopU1 that targets the RNA-binding protein GRP7 to manipulate the defense transcriptome (Fu et al., 2007; Nicaise et al., 2013).

Manipulation of host defense by virulence effectors has led to the evolution of a second surveillance layer of active defenses termed effector-triggered immunity (ETI). This layer is composed of intracellular immune receptors termed resistance (R) proteins that detect the presence of microbial effectors either through direct interaction or by detecting modifications made to host proteins (Figure 1.2; Jones & Dangl, 2006; Rafiqi et al., 2009). The discovery of indirect detection led to the “guard hypothesis”, in which specific effector targets are termed “guardees” and the R proteins are termed the “guards” (van
der Hoorn & Kamoun, 2008). The guarding R protein is triggered by perturbations in cellular homeostasis induced by effectors through biochemical modifications of host proteins or by the removal of specific host proteins via protein degradation. An example of the interaction between pathogenic effectors and R proteins is provided by the defense complex involving RPM1-INTERACTING PROTEIN4 (RIN4), a regulator of basal defense (Kim et al., 2005; Mackey et al., 2002; Wilton et al., 2010). It is guarded by two R proteins, the CC-NB-LRRs RESISTANCE TO PSEUDOMONAS SYRINGAE PV. MACULICOLA1 (RPM1), which confers dual resistance against the bacterial effectors avrRpm1 and avrB (Grant et al., 1995; Mackey et al., 2002), and RESISTANCE TO PSEUDOMONAS SYRINGAE2 (RPS2) which indirectly detects the effector avrRpt2 (Axtell & Staskawicz, 2003; Bent et al., 1994; Mindrinos et al., 1994). AvrRpm1 and avrB hyperphosphorylate RIN4 and enhance its activity, and these allosteric modifications are directly detected by RPM1 (Mackey et al., 2002). RPS2 on the other hand indirectly detects the avrRpt2-dependent degradation of RIN4 (Mackey et al., 2003). That a single protein is guarded by at least two R-proteins, and targeted by at least 3 pathogen effectors, emphasizes how pathogens target a small subset of important defense regulators. But the ongoing arms race also means that at least some pathovar specific effectors are not recognized in certain ecotypes, as is the case for the Pseudomonas effector Hop2Fpto that also targets RIN4 (Wilton et al., 2010).
Avirulence effectors detected by the host trigger a strong resistance response termed the hypersensitivity response (HR). HR is a stronger immune response than that triggered by MTI, and is usually followed by a rapid and localized area of cell death surrounding the point of pathogen entry. This form of cell death is designed both to kill host cells and obligate biotrophic pathogens that depend on the former, but also to trigger a massive release of anti-microbial compounds (Figure 1.3; Chisholm et al., 2006; Greenberg & Yao, 2004; Hatsugai et al., 2009; Hofius et al., 2007).

The general architecture of an R protein is a nucleotide-binding domain followed by a leucine-rich repeat domain (NB-LRR), with a variable signaling structure at the end that belongs to either the coiled-coiled (CC) or Toll and Interleukin-1 Receptor homology (TIR) family (Collier & Moffett, 2009). The LRR domain appears to be involved in interaction with recognition specificity, but at the same time to be an auto-inhibitory structure that
folds down on the NB domain. Upon detection, the LRR domain shifts away from the NB domain, allowing its catalytic core to hydrolyze nucleotides and changing the state of the signaling domain so it becomes active (Takken & Tameling, 2009). In accordance with this model, dominant negative R proteins have been identified with single point mutations in the NB domain (Palma et al., 2010). Interestingly, it appears that there are distinct signaling nodes downstream of the CC- and TIR-domains. This is surprising, given that activation of members of either R protein families most often result in the execution of a strong cell death response, to deter biotrophs (Elmore et al., 2011; Hofius et al., 2009a).

Unsurprisingly, some necrotrophs have evolved to exploit this cell death response and deliberately trigger HR cell death to gain access to nutrients (Govrin & Levine, 2000; Govrin et al., 2006; Lorang et al., 2012; van Kan, 2006). Also, the continued co-evolution of pathogens and plants has resulted in pathogen-derived effectors able to suppress ETI (Lindeberg et al., 2012).

1.4 Regulation of defense signaling

Although the level of complexity is high, general signaling events are shared against different attackers, which enables the plant to respond within minutes upon pathogen detection. Plant cells use complex signaling networks, and given the large number of potential defense-associated PRRs in the plasma membrane, it is not surprising that there is an apparent overlap in the signaling downstream of elicitor recognition. Both FLS2 and EFR form complexes with the receptors BAK1 and BAK1-LIKE1 (BKK1) and use them as signal enhancers or co-receptors. This is followed by early, similar bursts of reactive oxygen species (ROS), along with the opening of ion channels to allow flux across the

Figure 1.3: Example of an induced HR-associated cell death lesion (Right) 3 days after infection with Pst DC3000 avrRpm1 in Col-0 WT.
plasma membrane, with Ca2+ being one of the most important intracellular second messengers. Further downstream, signaling converges on MPK cascades that activate transcription factors (TFs), especially of the WRKY family (Roux et al., 2011; Zipfel et al., 2006; Jeworutzki et al., 2010; Asai et al., 2002; Rasmussen et al., 2012; Sun et al., 2013; Shi et al., 2013; Chinchilla et al., 2007; Felix et al., 1999). Similar features are also observed for other MAMPs such as bacterial derived peptidoglycans (PGNs) and chitin, and even viruses (Gust et al., 2007; Wan et al., 2008; Korner et al., 2013). The transcriptional response is also very similar for plants treated with either PGNs, EF-Tu, flg22 (Flagellin peptide) or chitin, indicating that the defense compounds produced share significant target overlap (Zipfel et al., 2004; Zipfel et al., 2006; Gust et al., 2007; Wan et al., 2008; Korner et al., 2013).

There is significant communication between different nodes in defenses, with downstream events shared between MTI and ETI (Navarro et al., 2004; Tsuda et al., 2008; Tsuda et al., 2009; Qi et al., 2011). A central mediator of both MTI and ETI is a protein of unknown function with limited homology to eukaryotic lipases, ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), that acts as a central positive regulator of basal resistance against biotrophs and hemi-biotrophs (Falk et al., 1999; Wiermer et al., 2005). It complexes with both PHYTOALEXIN DEFICIENT4 (PAD4) and SENESCENCE-ASSOCIATED GENE101 (SAG101), and is shuttled across the nuclear membrane depending on activation status (Feys et al., 2005; Feys et al., 2001; Wiermer et al., 2005; Wirthmueller et al., 2007; Garcia et al., 2010; Wiermer et al., 2012; Heidrich et al., 2011; Rietz et al., 2011). The role of EDS1 as an essential component in defense is seen by its requirement in the regulation of stomata closure and phytohormones, ROS production, and R protein responses (Ochsenbein et al., 2006; Wang et al., 2013; Brodersen et al., 2006; Parker et al., 1996; Falk et al., 1999). Thus, it comes as no surprise that EDS1 itself is an effector target, and that plants lacking EDS1 are highly susceptible to infection (Bhattacharjee et al., 2011; Aarts et al., 1998; Parker et al., 1996; Heidrich et al., 2011). EDS1 seems to be indispensable for TIR-NB-LRR triggered resistance (Falk et al., 1999). This is in contrast to many CC-NB-LRR type R proteins, of which a subset are instead primarily dependent on the NONRACE-SPECIFIC DISEASE RESISTANCE1 (NDR1) protein (Aarts et al., 1998). NDR1 is a plasma membrane associated integrin-like protein which interacts directly with RIN4, which is unsurprising,
given its requirement during RPS2 and RPM1 triggered immunity (Aarts et al., 1998; Coppinger et al., 2004; Day et al., 2006). As with EDS1, its molecular function is currently unknown but it may be involved in maintaining cell wall adhesion (Knepper et al., 2011).

During initial phases of defense activation, the three phytohormones salicylic acid (SA), jasmonate (JA) and ethylene (ET), regulate signaling networks in antagonistic or synergistic ways (Robert-Seilanianetz et al., 2011; Bari et al., 2009). Depending on the pathogen and its infection strategy, the timing and composition of active hormones varies. In genera, JA and ET defenses are activated in response to necrotrophic pathogens, while SA signaling is activated by biotrophs (Robert-Seilanianetz et al., 2011). A major aspect of the EDS1/PAD4 complex is regulation of the phytohormone SA, where it acts both in initial accumulation, but also as an enhancer through a positive feedback loop, amplifying the defense response of both MTI and ETI (Zhou et al., 1998; Falk et al., 1999; Feys et al., 2001; Shah et al., 2003; Wiermer et al., 2005; Venugopal et al., 2009; Vlot et al., 2009; Jirage et al., 1999; Tsuda et al., 2008). Several genes are involved in the synthesis of SA from chorismate and its translocation from the chloroplast during infection, including the transporter SA INDUCTION-DEFICIENT1 (SID1/EDS5) and the ISOCHORISMATE SYNTHASE1 (ICS1/SID2/EDS16), and both corresponding mutants show increased susceptibility to pathogens (Wildermuth et al., 2001; Nawrath et al., 1999; Serrano et al., 2013). This increased susceptibility is thought to derive primarily from a lack of expression of pathogenesis-related genes (PR), which is also observed when SA is degraded by introducing the bacterial SA hydroxylase NahG (Gaffney et al., 1993; Delaney et al., 1994; van Loon et al., 2006; Yamamoto et al., 1965).

SA has also been shown to be required for priming distal tissues against pathogens, by activating systemic acquired resistance (SAR), to combat potential secondary infections (Fu et al., 2013). SAR is an induced state of broad range resistance characterized by transcriptional reprogramming in systemic tissues, shifting resources from a purely vegetative state to one that has an active defense (Boatwright et al., 2013; Gruner et al., 2013). SAR can be triggered both by ETI and PTI, and the extent of SAR priming in distal tissues does not seem to be dependent on ETI-triggered cell death, but instead on the strength of the local immune response (Mishina et al., 2007). Exogenous application of SA
is sufficient to establish SAR, which is also mimicked by transgenic over-expression of a bacterial isochorismate synthase (White et al., 1979, Mauch et al., 2001). Conversely, introducing the hydroxylase NahG disables SAR induction (Delaney et al., 1994). Treatment of transgenic plants expressing NahG with SA analogs, such as benzothiadiazole (BTH) or 2,6- dichloroisonicotinic acid (INA), is sufficient to reinstate SAR due to the inability of NahG to degrade these compounds (Lawton et al., 1996). While SA itself is not the mobile signal for inducing SAR, it is required for the induction of the initial signal, and as an enhancer when the mobile signal reaches distal tissues (Gruner et al., 2013; Fu et al., 2013). Several compounds have been proposed as the mobile signal for inducing SAR including methyl-SA, JA, abietane diterpenoid dehydroabietinal (DA), glycerol-3-phosphate (G3P) and azelaic acid (Aza), but none have been confirmed as the bona fide mediator. Interestingly, the lipid transfer protein DEFECTIVE IN INDUCED RESISTANCE1 is required for the function of both G3P, DA and Aza, so the mediation of the signal may rely on multiple compounds that all enhance each other when they reach systemic tissues (Fu et al., 2013). Although the exact signal is still unknown, it has been established that the vascular tissue acts as the primary transport system for the signal (Tuzun et al., 1985; Kiefer et al., 2003).

The family of NON-EXPRESSOR OF PR-GENES (NPR) act as transducers of the hormonal SA signal to the nucleus, both during local infections and during SAR, regulating several WRKY and TGA TFs, ultimately leading to changes in the expression of more than 2000 defense-related proteins (Wang et al., 2006; Despres et al., 2000; Fu et al., 2012). NPR1/NIM1 has long been viewed as a key regulator of SA mediated signaling, being essential for both local defense initiation and SAR activation (Cao et al., 1997; Wildermuth et al., 2001; Delaney et al., 1995; Kinkema et al., 2000). Research has not been able to show interaction between NPR1 and SA, and only recently has NPR3 and NPR4 been identified as receptors for the hormone. Surprisingly, the npr3 npr4 double mutant shows enhanced resistance, partially dependent on NPR1, but also SAR initiation deficiency, which has led to the development of a complex model (Fu et al., 2012; Zhang et al., 2006). In the steady state condition when SA levels are low, NPR1 is constitutively degraded by the proteasome in a CUL3 E3 ligase dependent manner in which NPR4 act as
an adapter. During an infection when SA levels rise, NPR4 acts as a SA receptor, hindering its ability to act as an adapter for CUL3-mediated ubiquitination and subsequent degradation of NPR1. This allows a subpopulation of the NPR1 pool to move to the nucleus and activate defense genes through interaction with TFs. At the same time, increasing SA levels are also mimicked in systemic tissues due to the SAR signal, allowing NPR1 to prime distant tissues. During ETI, even higher local levels of SA are present, allowing NPR3 to act as a second, lower affinity SA receptor and NPR1 adapter, also increasing NPR1 turnover. This results in a brief window of NPR1-dependent transcriptional activation (Fu et al., 2012; Enyedi et al., 1992; Spoel et al., 2009). Surprisingly, Fu et al. (2012) were unable to detect interaction between NPR1 and SA, which is contrast to the work by Wu et al. (2012). It was proposed that differences in the technical setup were the reason for the different results, and that NPR1 and SA interact in a highly unstable manner, allowing NPR1 to promote structural changes through rapid detection of the hormone (Wu et al., 2012). As NPR1 is also sensitive to changes in redox environment, as it contains several sensitive disulphide bonds allowing it to oligomerize, it is also possible that assay variations may account for the observed differences, and that SA-dependent responses are fine tuned to various conditions (Kinkema et al., 2000; Mou: 2003vx, Boatwright et al., 2013; Kaltdorf et al., 2013). Notably, NPR1 acts as both a negative and positive regulator of R protein triggered cell death, so its exact role in defense regulation remains to be determined (Fu et al., 2012; Rate et al., 2001). It should be noted that while SA is a primary signal component of defense signaling, there are also EDS1- and SA-independent defense signaling pathways (Venugopal et al., 2009; An et al., 2011).

While many PR proteins have direct antimicrobial activities, others are involved in signaling and other aspects of defense. Due to pathogen-induced stress, many of the products are expressed to maintain cellular homeostasis (Vitale et al., 2008). This is especially apparent in that NPR1 up-regulates genes required for the secretory pathway which is vital for thickening the cell wall and secretion of antimicrobial proteins (Wang et al., 2005). Enhanced secretion of defense-related compounds can also be NPR1-independent, but regardless of the regulatory pathways that trigger the response, most if not all PR proteins go through the endoplasmatic reticulum (ER) before being secreted.
(Watanabe et al., 2013; Eichmann et al., 2012). The intensified activity in the ER during the initial phases of an infection, or during exposure to abiotic stressors, is countered by triggering a stress response known as the unfolded protein response (UPR) to combat the aggregation of misfolded proteins (Howell et al., 2013; Fanata et al., 2013). If nascent peptides start to accumulate in the ER lumen, hydrophobic interactions may cause them to form aggregates, hindering further exit from the organelle. To avoid this, molecular chaperones such as BINDING PROTEIN (BiP) interact with and stabilize proteins (Eichmann et al., 2012). Two different sensors have been described in plants so far, both of which transmit a signal to the nucleus by activating bZIP TFs (Howell et al., 2013). In the first system, a high abundance of unfolded proteins results in BiP disassociation from the transmembrane proteins bZIP17 and bZIP28, which permit them to cluster in the membrane. This grouping allows them to move to the Golgi through vesicular movement, where local proteases cleave their cytosolic domains, allowing them move to the nucleus where they activate stress-responsive genes (Howell et al., 2013; Eichmann et al., 2012). The second sensor is believed to rely on the ribonuclease activity of membrane bound INOSITOL-REQUIRING ENZYME1a and 1b (IRE1a/b) that is induced upon detection of an abundance of misfolded proteins in the ER lumen. Under unstressed conditions the bZIP60 TF is located in the ER membrane, but upon activation, IRE1 splices the bZIP60 mRNA transcript to a truncated form without its transmembrane domain, allowing newly translated forms of the TF to move freely to the nucleus (Iwata et al., 2008; Iwata et al., 2005; Nagashima et al., 2011; Deng et al., 2011; Mishiba et al., 2013; Moreno et al., 2012). Upon reaching the nucleus, TFs from both pathways converge to regulate the expression of many similar stress-associated genes, especially ER chaperones such as BiP and enzymes required for proper folding (Iwata et al., 2008; Liu et al., 2010). Alongside the UPR, the ER-associated degradation (ERAD) system steps in to remove misfolded proteins through translocation to the cytosol and subsequent ubiquitination for proteasomal degradation (Brandizzi et al., 2003; DiCola et al., 2005; DiCola et al., 2001; Liu et al., 2011; Howell et al., 2013). As the ER starts to swell due to the stress load, the plant cell also actively start to degrade the organelle to combat protein accumulation (Liu et al., 2012). But if the UPR is not capable of maintaining structural integrity, prolonged ER stress will
ultimately lead to cell death (Qiang et al., 2012; Zuppini et al., 2004; Ishikawa et al., 2011). This is especially apparent when artificially triggering cell death by treating plants with compounds that specifically stress the ER, such as tunicamycin (TM), an N-glycosylation inhibitor, or brefeldin A, a protein trafficking inhibitor (Crosti et al., 2001).

1.5 Cell death

Most multicellular species use programmed cell death (PCD) to shape their anatomy by removing unwanted tissue or individual cells. PCD death can also be caused by various forms of external biotic and abiotic stressors, both as a direct pro-death response but also as a pro-life mechanism to maintain homeostasis in surrounding tissues (Baehrecke et al., 2002; Fulda et al., 2010). Plants do not differ from animals much in these aspects. Some prominent examples of plant PCD are the developmentally associated formation of vascular tissue or the morphing of leaf shapes (van Doorn et al., 2005). While these examples have been well described morphologically, molecular insight into both the perception, regulation and execution of cell death pathways in plants is still in its infancy (van Doorn et al., 2011), in contrast to the current view of mammals where several distinct cell death pathways have been recognized (Nikoletopoulou et al., 2013). Whereas some of them are still only poorly described, such as ferroptosis, a distinct mechanism that relies on iron pertubations (Dixon et al., 2012) or mitoptosis, a kind of mitochondrial suicide pathway (Jangamreddy et al., 2012; Tinari et al., 2007), it is still not entirely clear if these are independent pathways or subsets under the more generally accepted forms of PCD such as apoptosis or autophagy. It was believed that the different PCD pathways were mutually exclusive, but it is now clear that while both the inducing factors and the state of the cellular system help shape the response, some apparent cross-talk occurs. These interactions make it hard to specifically define and morphologically describe individual pathways (Nikoletopoulou et al., 2013).

Necrosis has long been viewed as the alternative to PCD in situations where acute stress or overwhelming trauma end in a series of uncontrolled actions resulting in unordered cellular collapse and cell death. It is considered an all or nothing response, and
has been defined by certain morphological markers such as nuclear and organelle swelling, and plasma membrane rupture followed by potential local tissue damage caused by leaking cellular debris (Edinger et al., 2004). Notably, several cases of necrosis in mammals seem to be induced and regulated as other PCD pathways. This form of recruited necrotic PCD has been termed necroptosis, but has not been described in plants (Galluzzi et al., 2008; Wu et al., 2012; Nikoletopoulou et al., 2013).

In contrast to necrosis, apoptosis is the best described PCD pathway in mammals. In some cases it is synonymous with PCD, and variations of it have been documented in a wide array of organisms such as Caenorhabditis elegans (C. elegans) and yeast (Hengartner et al., 1994; Frohlich et al., 2000). Apoptosis was first described by Kerr et al. (1972) and is accompanied in mammals by cellular shrinkage and detachment, along with condensation of chromatin, nuclear fragmentation, budding from the plasma membrane and gradual exocytosis of apoptotic bodies containing cytoplasmic content and organelles (Kerr et al., 1972, Elmore et al., 2007). At the molecular level apoptosis can be activated by external stimuli through transmembrane death receptors such as TUMOR NECROSIS FACTOR-α, TNF-RELATED APOPTOSIS INDUCING LIGAND and Fas that allow the recruitment of a larger complex of proteins that acts as adapters for the recruitment of the protease caspase-8. This protease can then mediate proteolysis of BH3-INTERACTING DOMAIN DEATH AGONIST, a pro-apoptotic member of the Bcl-2 family, which in turn recruits and initiates the embedding of Bak and Bax, two other Bcl-2 family members, into the mitochondrial outer membrane (MOO). The pore-like structure of Bak and Bax oligomers in the MOO results in the export of a number of death-promoting proteins including cytochrome C. In the cytosol, cytochrome C allows the establishment of the apoptosome in which CASPASE-9 acts instigates a cascade that results in the activation of several members of the proteolytic caspase family. As a consequence, these proteases activate or degrade specific proteins required for maintaining cellular integrity. This serial activation of cytotoxic caspases is a major feature that distinguishes apoptosis from other PCD pathways (Nikoletopoulou et al., 2013; Taylor et al., 2008). This route of apoptotic activation has been called the extrinsic pathway, due to the external activation, and one alternative is through import of the protease GRANZYME B, which also acts on BID, in
the same way as described above for CASPASE-8. Another scenario is the intrinsic pathway in which internal stimuli or stress can activate members of the BH3-only family, which in turn trigger Bax/Bak pore formation in the MOO (Nikoletopoulou et al., 2013; Taylor et al., 2008).

Plant cells do not seem to have an apoptotic pathway as they don’t produce apoptotic bodies and have no caspases. However, when cell death is induced, they do share some features reminiscent of those observed in mammals such as degradation of DNA and detachment from substrates. In addition, plant cells have a cell wall which would hinder the exocytosis of apoptotic bodies and, since they lack phagocytic cells, they do not have an immediate way of removing this cellular debris which may be damaging to surrounding tissues (Reape et al., 2008). As plants lack caspases, it is unclear if they are capable of initiating the destructive cascade at the end of the apoptotic pathway. Instead, plants possess meta-caspases which have been shown to be involved in cell death regulation, but which have different substrate specificities than caspases (Coll et al., 2010; Vercammen et al., 2004; Tsiatsiani et al., 2013). A possible set of orthologs may be the vacuolar processing enzymes (VPEs), which possess caspase-like activity, indicating that plant analogs may have evolved to take the role of caspases (Hara-Nishimura et al., 2005; Hatsugai et al., 2004). In addition to the activity of these proteases, cathepsins appear to promote HR PCD, indicating that they are also involved as pro-death executors (Hofius et al., 2009; McLellan et al., 2009; Gilroy et al., 2007). Surprisingly, the plant proteasome was also found to exhibit caspase-like activity and to be involved in cell death through perforation of the tonoplast (Vacuolar membrane), indicating that phyto-specific cell death pathways also exist (Hatsugai et al., 2009; Woltering et al., 2010).

The vacuole appears to be central in plant PCD, which is not surprising given that it usually occupy more than 90% of the cells volume (Wink et al., 1993). It has been suggested that vacuolar and non-vacuolar (necrotic) cell death are distinguishable in plants. This may explain why, in the absence of apoptosis does not exist, loss of vacuole integrity may be sufficient to cause cellular collapse (van Doorn et al., 2011). As part of the HR induced upon detection of microbial effectors, the associated PCD is required to be strong enough to ensure a localized path of dead cells. At the same time, collapse of the vacuole will flood
the apoplast (intercellular space) with anti-microbial compounds and lower the external pH, which would also explain the central role of the vacuole in plant PCD (Hatsugai et al., 2009). Moreover, crosstalk between different cell death pathways in mammals is well documented such that blocking of one pathway can enhance other pathways, leading to similar outcomes (Nikoletopoulou et al., 2013). Even in this view, it still unclear why there seem to be different pathways for the regulation and execution of cell death, as seen for EDS1 and NDR1, and the observed additive effect on the strength of the response by different PCD executioners (Hofius et al., 2009; Hofius et al., 2009). However, it is clear that ROS production and transmembrane ion fluxes are a requirement upon effector detection for establishing a proper PCD response in plants (Nanda et al., 2010; Cheval et al., 2013).

### 1.6 Autophagy

Autophagy is a catabolic process for degrading cytoplasmic content, including whole organelles. It is present in all eukaryotic species. Autophagy is induced by various stimuli and is involved in recycling of nutrients during starvation (Robaglia et al., 2012), removal of damaged organelles (MacVicar et al., 2013), counterbalancing nuclear stress (Mijaljica et al., 2013), regulation of senescence (Goehe et al., 2013), and as a general response to various forms of stress (Ryter et al., 2013). Deviations in autophagic processes are seen in cancers as the process is crucial for maintaining cellular homeostasis, and it is therefore also widely considered a pro-life pathway (Kaushik et al., 2010; Lu et al., 2013). The process relies on the concerted action of autophagy-related gene products (ATG) that sequester cellular material in an acidic environment for subsequent degradation. In mammals this acidic compartment is the lysosome, whereas in yeast and plants it is the vacuole (Viotti et al., 2013; Bassham et al., 2006). Several variations of autophagy have been described in yeast and animals, and at least two of them are known to be present in plants: Micro-autophagy, in which the lysosomal or vacuolar membrane creates invaginations for direct uptake of cytosolic constituents, and macro-autophagy which acts through the formation of double membrane structures around the content to be degraded, and subsequent fusing of the outer membrane with the vacuole or lysosome (Yoshimoto et al., 2010). Macro-autophagy,
the best described process, will be the focus in this thesis and will be referred to as “autophagy”.

Molecular studies in yeast have provided us with a core set of more than 30 ATG genes required for the autophagic process (Yang et al., 2010; Lamb et al., 2013). The best described regulator of autophagy in mammals and yeast is the kinase complex TARGET OF RAPAMYCIN (TOR), an inhibitor of ATG1 and ATG13, both members of the complex that initiates autophagy. Upon amino acid starvation TOR itself is inhibited, triggering enhanced protein flux through the autophagic process, thus increasing turnover of proteins and other nutrients. In addition, inhibition of TOR lowers synthesis of new proteins, as it is also a positive regulator of protein translation (Noda et al., 1998; Lamb et al., 2013; Robaglia et al., 2012). Another prominent suppressor of TOR is ER stress, as heightened pressure on the expanding organelle is dependent on autophagy to degrade and restore ER functionality (Kaushik et al., 2010). ROS is also a potent inhibitor of TOR, while at the same time a trigger for the activation of the ATG6-VPS34 complex (Gibson et al., 2013). For a more thorough introduction to individual ATGs and regulatory nodes involved in autophagy in mammals/yeast and plants, see the reviews from Stanley et al. (2013) and Bassham et al. (2006) respectively.

Based on assays from mammals and yeast, the autophagic process can be divided into three different phases in which complexes composed of specific ATGs are involved in each step (Figure 1.4). In the initiation phase, key components are recruited to form the isolation membrane or phagophore. ATG1-ATG13-ATG17 are first recruited and serve as the basis for the remaining components, while coordinating the fusion of early membrane material. This is followed by ATG9-containing vesicles, which may originate from the trans-Golgi network (TGN), providing the initial membrane constituents to form the isolation membrane. Then VPS34-ATG6-ATG14-VPS15 are recruited and form a phosphoinositol 3-kinase (PI3K) complex to produce phosphatidyl-inositol-3-phosphate (PtdIns(3)P) in the membrane, which is crucial for maturation of the finished double-membrane structure termed an autophagosome. Through interaction with PtdIns(3)P, ATG9 then forms a complex with ATG2-ATG18 which mediates its recycling (Simonsen et al., 2009; Stanley et al., 2013; Gallagher et al., 2013; Lamb et al., 2013). In the second
nucleation or elongation phase, ATG3, ATG7 and ATG10 form two parallel ubiquitin-like conjugation systems that associate with the membrane and form ATG5-ATG12-ATG16 that directs the conjugation of ATG8 to phosphatidylethanolamine (ATG8-PE) in the membrane. Thus, as the autophagosomal membrane expands, cytoplasmic content is enveloped in an ATG8 coated double membrane. ATG8 is therefore also often used as a marker for assaying autophagosome formation with fluorescent tags or electron microscopy and, in part, for verifying increased protein flux through the autophagic process (Tabata et al., 2013; Lamb et al., 2013). How and where the majority of lipids for the formation of the autophagosomal membrane are derived from is still hotly debated. Several groups have provided evidence of lipids being transported from both the Golgi apparatus, recycling endosomes, plasma membrane, mitochondria and the ER (Hailey et al., 2010; Axe et al., 2008; Tooze et al., 2013). Recently it was suggested, at least in starved mammal cells, that autophagosomes form at mitochondria-ER contact sites, which can explain at least some of the different observations in the literature, as this is a location where lipid exchange between the two organelles happens (Hamasaki et al., 2013; Rowland et al., 2012; Ge et al., 2013). In yeast, the site of formation of the isolation membrane is at a single location called the pre-autophagosomal structure, situated next to the vacuole (Stanley et al., 2013). It is currently unknown where the initial complex formation happens in plants, but since they lack a homolog of ATG14 it is unlikely to be at the ER membrane, since ATG14 serves as an anchor in the forming isolation membrane (Hamasaki et al., 2013). At the end of the process, ATG8 is deconjugated from the external autophagosomal membrane to ensure complete release of ATG proteins and proper shuttling of the fully formed vesicle to the lytic compartment (Nair et al., 2012). In the third phase the fully formed autophagosome is transported to the lytic compartment where the outer membrane fuses with the vacuolar or lysosomal membrane and releases the inner membrane with its content into the lumen where proteases and hydrolases mediate degradation (Lamb et al., 2013). It is still unclear if autophagosomes fuse with the central vacuole in plants, or with a separate smaller, lysosome-like vacuole (Viotti et al., 2013).
Cargo sequestered by the autophagosome can be selected specifically through the binding of ubiquitinated substrates to cargo-receptors, or non-selectively by engulfing bulk cytoplasm. The cargo-receptors, or adapters, associate with the inner autophagosomal membrane by binding to ATG8 and, through their interaction with ubiquitin residues on aggregated proteins destined for degradation, target specific proteins for degradation. Two different cargo adapters have been identified in mammals, p62 and NBR1 (Pankiv et al., 2007; Lamark et al., 2009; Bjorkoy et al., 2005). Interestingly, in Arabidopsis there is a single NBR1 homolog that shows similarity to both mammalian NBR1 and to p62. So far it is unknown what specific cargo it targets, although it seems to be involved in responses to abiotic but not biotic stress (Svenning et al., 2011; Zhou et al., 2013; Floyd et al., 2012). The selective process is also known to target organelles, such as degradation of dysfunctional mitochondria, or parts of the ER during severe stress (MacVicar et al., 2013; Kaushik et al., 2010).

Insight into the autophagic machinery in plants is still limited but, through complementation of yeast ATG knockout models, it has been confirmed that plant orthologs share similar functions, and that the overall mechanism is present (Yoshimoto et al., 2010; Liu et al., 2012; Bassham et al., 2006). Especially the TOR complex appears to be functionally conserved in plants, and it has been observed to be involved in the regulation of osmotic stress responses, hormone signaling, development and growth (Deprost et al.,

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Figure 1.4: Core complexes and conjugation pathways required for autophagy, with genes shown that are present in Arabidopsis.
2007; Xiong et al., 2012; Ahn et al., 2011; Deprost et al., 2005; Menand et al., 2002; Agredano-Moreno et al., 2007; Mahfouz et al., 2006; Ren et al., 2011). In addition, several autophagic mutants have been described in Arabidopsis in which ATG null alleles (atg) result in a lack of autophagic bodies (Doelling et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005). As autophagy is so closely involved in amino acid and lipid turnover, it is surprising that autophagy-deficient mutants seem to develop normally under nutrient-rich and stress-free conditions. This indicates that autophagy may not be an essential process during development. Under most conditions however, autophagy-deficient mutants display early senescence, as visible by loss of chlorophyl and collapsing leaves in a shorter timeframe compared to that of wild type (WT) plants (Yoshimoto et al., 2010). As it is a general response to stress in plants, it may indicate that autophagy is only required during deviations in cellular homeostasis (Yoshimoto et al., 2009; Xiong et al., 2007; Liu et al., 2012; Han et al., 2011; Howell et al., 2013). In apparent acknowledgement, exposure of autophagy-deficient mutants to stress, such as heat, accelerates the accumulation of ubiquitinated proteins (Zhou et al., 2013).

During both Arabidopsis and Populus development, autophagy has been observed to function as a cell death pathway involved in shaping tracheary elements (Kwon et al., 2011; Kwon et al., 2010). Whether it functions as a pro-death or pro-life response during pathogen-induced HR is still not clear, as there are conflicting observations during avirulent infections in autophagy-deficient mutants (Hofius et al., 2011). Several groups have reported spreading of HR lesions outside of the infected area in ATG knockout plants, positioning autophagy as a pro-life response (Yoshimoto et al., 2009; Liu et al., 2005; Patel et al., 2008). Yoshimoto et al. (2009) further demonstrated a correlation between early senescence in atg mutants and increases in SA levels, both of which could be rescued by introducing mutations in genes involved in SA signaling, such as NPR1 and SID2, or by introducing NahG. These mutations also rescued the lesion spread post infection, leading the authors to propose that autophagy negatively regulates SA signaling, through NPR1, limiting senescence and immunity-associated cell death. In apparent contrast, our group has observed that autophagy is required as a cell death pathway during immediate HR development (Hofius et al., 2009). In agreement with our observations, it was discovered...
that the GTPase RabG3b positively regulates autophagy during immunity-associated cell
death (Kwon et al., 2013). These conflicting observations will be the main focus of the
results section 3.3.

1.7 Lesion mimic mutants

In order to retain tissue homeostasis, it is necessary to keep close control over cell death
pathways. Too stringent control and cancers may develop, and too little may result in
patches of dead cells. Due to genetic defects in regulatory nodes involved in cellular
homeostasis, physiological perturbations may result in spontaneous cell death induction.
In addition, as an activated HR usually employs a very strong all-or-nothing induction of
cell death, R proteins, the proteins they guard and downstream PCD regulators need to be
carefully regulated and kept from non-deliberate activation. Therefore, if spontaneous cell
death occurs in mutants in the absence of stimuli, they may be designated as lesion mimic
mutants (LMMs). This has led to the designation of several mutants often called
ACCELERATED CELL DEATH (ACD) or LESION SIMULATING DISEASE (LSD)
mutants. They can generally be classified into two groups: those that show constitutive
lesion formation in specific or all tissues, or those incapable of suppressing the extent of an
activated cell death response (Dietrich et al., 1994; Moeder et al., 2008; Lorrain et al., 2003).
The uncontrolled cell death seen in LMMs often bears strong resemblance to that observed
during the HR, and is often supported by a dependence of SA- or R protein signaling
components (Wiermer et al., 2005). These HR-like prerequisites position LMMs as tools for
unraveling the nature of PCD and its upstream components, along with the basic
mechanisms of plant defense pathways. With around 150 NB-LRRs currently identified in
Arabidopsis, it is possible many more LMMs will be identified (Meyers et al., 2003).

Prominent examples of characterized LMMs are lsd1 and acd5. lsd1 is a conditional
recessive mutant that shows hyper-responsiveness to environmental stress such as
drought, cold temperatures, ROS exposure, BTH treatment or long day light (16 hours a
day), most of which induce lesion propagation and upregulation of SA- and EDS1-
dependent PR proteins. It also exhibits increased resistance to pathogens even in the
absence of external stimuli (Huang et al., 2010; Jabs et al., 1996; Wituszynska et al., 2013; Torres et al., 2005; Dietrich et al., 1994; Kliebenstein et al., 1999; Aviv et al., 2002; Rusterucci et al., 2001; Jabs et al., 1996). The LSD1 gene encodes a zing-finger protein with homology to mammalian transcription factors. LSD1 localizes to the nucleus, indicating that it may be directly involved in gene transcription (Dietrich et al., 1997; He et al., 2011) At the same time, LSD1 interacts with catalases that convert H₂O₂ to water and oxygen, and appears to regulate their function during PCD. This indicates that it is central for defense regulation, and thus a target for pathogen effectors (Li et al., 2013). In accordance with this, the CC-NBR-LRR ADR1-L2 suppresses the runaway cell death phenotype in lsd1 mutants of both Columbia-0 (Col-0) and Wassilewskija (WS) ecotypes (Bonardi et al., 2011). The other example of a recessive LMM, acd5, is characterized by spontaneous disease-like lesions that emerge several weeks post germination. So far no R protein has been found as the trigger involved in its mutant cell death phenotype. ACD5 encodes a ceramide kinase and is therefore involved in sphingolipid metabolism. Interestingly, the balance between ceramide and ceramide-1-phosphate (C1P) is involved in the choice between life and death signaling in both mammals and plants (Liang et al., 2003; Cuvillier et al., 1996; Gomez-Munoz et al., 1997). As extraneous ceramide triggers cell death in plant protoplasts, it could indicate that the cause of cell death is due to build up of non-phosphorylated ceramide. Compared to WT, acd5 appears to be slightly more susceptible to virulent Pseudomonas syringae pv. maculicola and Pst but, surprisingly, it also accumulates SA and PR proteins to higher levels than WT. This indicates an uncoupling between resistance and SA-dependent cell death signaling (Greenberg et al., 2000). Interestingly, acd5 is not the only LMM that displays this kind of separation of SA-responses, as is has also been observed in other mutants such as the DEFENSE, NO DEATH1 (DND1) and DND2 mutants, which encode the CYCLIC NUCLEOTIDE-GATED CHANNELs (CNGC), CNGC2 and CNGC4 (Clough et al., 2000; Jurkowski et al., 2004; Yu et al., 1998).
1.8 ACD11, FAPP2 and sphingolipids in cell death

ACD11 is an LMM that displays a strong HR-like cell death and immunity-associated phenotype shortly after germination, which is ultimately lethal at early stages of development (Figure 1.5). Similar to the LMMs described in section 1.7, it is highly dependent on SA signaling, as the introduction of NahG completely suppress the phenotype. This makes an \textit{acd11 NahG} double mutant a valuable tool for examining PCD pathways, as it is possible to reinstate the heightened defense state at any stage during its development by extraneous application of BTH or INA. The cell death phenotype appears to be triggered through an NPR1-independent pathway, while this is not the case for the defense-related phenotype, as it is almost fully reversed by the introduction of \textit{npr1} into \textit{acd11} (Brodersen \textit{et al.}, 2002; Brodersen \textit{et al.}, 2005). Not much is known about the function of ACD11 but it interacts in yeast-2-hybrids with PRA7 and PRA8, two proteins homologous to human PRENYLATED RAB ACCEPTOR1 (PRA1) involved in vesicle transport from the Golgi (Petersen \textit{et al.}, 2009). \textit{At}ACD11 shows homology to human GLYCOLIPID TRANSFER PROTEIN (GLTP) and also catalyze the transfer of sphingosine, but not ceramide or galactosylceramide between membranes in vitro (Brodersen \textit{et al.}, 2002). The \textit{het-c} gene from the filamentous fungus \textit{Podospora anserina} also shows homology to \textit{AtACD11}. Interestingly, this fungus induces autophagy during incompatible interactions to prevent heterokaryosis (Pinan-Lucarre \textit{et al.}, 2003; Saupe \textit{et al.}, 1995). \textit{acd11}-triggered cell death also displays an up-regulation of autophagy, but it is unknown if this is as a pro-death executioner or as a stress response (D. Hofius, unpublished).

\textbf{Figure 1.5:} 4 weeks old Ler wild type plant (Left) compared to \textit{Ler acd11-1} (Right). Scale bar indicates 1 cm.
Sphingolipids are a large class of lipids with around 168 species in Arabidopsis alone, believed to be major components of the endomembrane system, the plasma- and the tonoplast membranes (Markham et al., 2007; Markham et al., 2013). Synthesis of new sphingolipids starts in the ER with production of amino alcohol long chain bases (LCBs), usually around 18 carbon atoms long, which gets acetylated with a 14-26 carbon atom fatty acid to form a ceramide. This basic structure forms the basis from which more complex sphingolipids are synthesized, such as through modification of the OH-group on the LCB with a phosphoryl group (Ceramide phosphates) or hexoses (Glycosylceramides), or by modification of chain length of either the fatty acid or the LCB. Each of the four major groups of plant sphingolipids, LCBs, ceramides, glycosylceramides or phosphoceramides, play individual roles, but are also working together and continuously converted back and forth between species (Markham et al., 2013; Pata et al., 2010; Zauner et al., 2010). Sphingolipid metabolism is therefore incredibly complex and sphingolipid homeostasis may be a prominent target for pathogen effectors, especially considering that deviations occur during the regulation of both ETI and PTI (Liang et al., 2003; Cuvillier et al., 1996; Gomez-Munoz et al., 1997; Peer et al., 2010; Pata et al., 2010). This also appears to be the case, as Fumonisin B₁ (FB₁), a sphingoid-like mycotoxin produced by several necrotrophic species, blocks SERINE PALMITOYLTRANSFERASE (SPT; A ceramide synthase), presumably by blocking the binding sites of sphingolipid precursors [Merrill Jr et al., 2001; Shi et al., 2007]. SPT is the first rate limiting step of sphingolipid synthesis and this induced metabolic shift results in accumulation of free sphingoid bases, mimicking conditions observed during avirulent infections (Abbas et al., 1994; Peer et al., 2010; Shi et al., 2007). In support of this, deviations in the homeostasis between free sphingoid bases and their metabolized forms has been shown to induce cell death (Shi et al., 2007; Chen et al., 2008; Lachaud et al., 2010). Other ceramide precursors, such as long chain bases and very long chain fatty acids also appear to be tightly regulated during HR cell death (Raffaele et al., 2008; Gan et al., 2009; Saucedo-Garcia et al., 2011). It has been shown that the levels of free LCBs regulate the production of ROS, and subsequently PCD through a VPE dependent pathway (Shi et al., 2007; Kuroyanagi et al., 2005). Interestingly, this PCD regulation is
independent of NPR1, imitating the genetic mechanism behind \textit{acd11}-associated PCD \cite{Asai2000, Brodersen2002}.

Apart from homology to HsGLTP, ACD11 is also quite similar to the C-terminal GLTP domain of mammalian \textsc{Four-Phosphate-Adaptor Protein2} (FAPP2), a protein involved in the formation of transport carriers from the TGN to the plasma membrane \cite{Godi2004}. Through binding of PtdIns(4)P by its N-terminal plekstrin homology (PH) domain, FAPP2 specifically regulates post-Golgi transport, presumably by assisting in membrane tubulation \cite{Godi2004, Cao2009}. In support of this proposed function, transport of glycophsphatidylinositol-anchored proteins, vesicular stomatitis VIRUS G PROTEIN (VSV-G), glycosaminoglycans and aquaporin-2 is partially blocked at the TGN upon FAPP2 knockdown by small interfering RNAs (siRNA; Vieira \textit{et al}., 2005; Yui \textit{et al}., 2009; D’Angelo \textit{et al}., 2007). This transport specificity is probably via the modulation of membrane composition, as FAPP2 appears to be required for the synthesis of more complex glycosphingolipids by binding simple glycosylsphingolipids or glycosylceramide and assisting in the transportation through the Golgi to sites where they are additionally modified \cite{D’Angelo2007}. In addition, FAPP2 interacts with members of the \textsc{Vesicle-Associated Membrane Protein-Associated Protein} (VAP). This is interesting as ACD11 also interacts with at least one members of the VAP family in plants, indicating that they may share similar functionality although they bind differently \cite{Mikitova2012, Petersen2009}. Apart from interfering with sphingolipid metabolism, siRNA knockdown of FAPP2 also increases sensitivity to the apoptosis-inducing factor FasL, indicating cellular stress. This is further supported by the observation that FAPP2 is upregulated in many cancer lines \cite{Tritz2009}. Interestingly, Skov et al. (manuscript in preparation) found that knockdown of FAPP2 in Jurkat cells leads to increased cell death and autophagy activation. As \textit{acd11}-induced PCD appears to mimic that of an HR and induces autophagy, and as mammalian and plant innate immunity share many features, it is possible that the role of FAPP2 and ACD11 in cell death share similar genetic components \cite{Nurnberger2004}. This possibility will be the basis of the results section 3.2.
1.9 ACD11 suppressor screen

To better understand *acd11*-associated cell death, a genetic screen for suppressors was carried out in our group using various mutagens (Ethane methyl sulfonate, γ-radiation and diepoxybutane). 3 million mutagenized Landsberg (Ler) *acd11 NahG* double mutants were treated with BTH in the M2 generation, and all surviving plants were assigned different complementation groups using linkage analysis. Around 250 putative mutants were identified along with alleles of EDS1 and PAD4 as a validation of its utility. The remaining mutants were divided into 11 recessive and 2 dominant complementation groups, shown that they were not defective in BTH uptake or response, and named laz mutants (From the biblical resurrection of Lazarus; Malinovsky *et al.*, 2010).

The first laz mutant characterized, *laz1*, was found to be involved in effector-triggered cell death. Mapping of *laz1* identified a mutation in a transmembrane protein of unknown function containing a DUF300 domain. It has homology to the human tumor suppressor TMEM34 and bile transporter Ost-α, but its function in *acd11*-associated cell death is currently unknown (Malinovsky *et al.*, 2010). *laz2* was found to encode the histone lysine methyltransferase SDG8, an epigenetic flowering time regulator through histone 3 modification. Transcriptome analysis of BTH-treated *acd11 NahG* and *laz2* *acd11 NahG* identified several target genes being down regulated in *laz2*, including several *R* genes. One of these was shown to be allelic with putative mutants in the largest laz complementation group including several dominant alleles. This *R* protein was named LAZ5 and found to encode a TIR-NB-LRR protein required to activate the *acd11* cell death phenotype in the Ler ecotype. Palma *et al.* (2010) found that LAZ2 is capable of regulating local expression through chromatin modification of the LAZ5 loci, thus confirming the hypothesis of ACD11 as a guarded protein (Palma *et al.*, 2010). The role of ACD11 as a guarded effector target is supported by the observation that *acd11* complemented with either HsGLTP and sphingosine transfer-deficient ACD11 can partially suppress the cell death phenotype. This indicates that ACD11 structural features are most important in triggering the cell death phenotype (Petersen *et al.*, 2008). The role of sphingolipids in LAZ5-triggered PCD will be discussed in the results section 3.1.
1.10 LAZ4 and the retromer complex

The remaining laz mutant, which is the focus of section 2.1 of this thesis, is laz4, a relatively weak suppressor of acd11-associated PCD (Figure 1.6). Part of a complementation group consisting of four EMS mutagenized alleles (E196.1, E209.3, E222.1 and E74.1), the laz4-1 allele was mapped to the lower arm of chromosome 1 between loci At1g75790 and At1g75970. Expression analysis of the BTH treated acd11 NahG transcriptome and subsequent sequencing revealed a single point mutation at an intron splice acceptor site in the 8th exon of At1g75850. This splice mutation may result in improper splicing of the intron from the precursor mRNA, which would make the translation machinery encounter a premature stop codon a short distance downstream, and thus synthesize a truncated version of the protein. At1g75850 contains 22 exons spanning more than 6 kilobases, and encodes one of three homologs of the VACUOLAR PROTEIN SORTING35 (VPS35), more specifically VPS35b, which is part of the multi-oligomeric retromer complex responsible for recycling of proteins from late endosomal compartments to the TGN (Figure 1.7; Oliviusson et al., 2006; Yamazaki et al., 2007; Kang et al., 2012; Nodzynski et al., 2013). Complementation analysis verified that the nature of the laz4-1 mutation was the cause of acd11-related PCD suppression, indicating a role of VPS35b in HR cell death. Single knockout mutants of the three VPS35 homologs (VPS35a, VPS35b and VPS35c) were then analyzed in Pst DC3000 avrRpm1 triggered cell death, but neither displayed any suppression compared to WT. This thesis will focus on the potential contribution of redundancy between VPS35 members in HR-associated PCD and resistance, in section 3.1 of the results.
In plants, the function of the retromer is still not well understood. It appears to have the same overall structure as that in yeast and animals in which VPS35 acts as a scaffold that interacts with the other two core components, VPS26 and VPS29, and with vacuolar sorting receptors (VSRs; Oliviusson et al., 2006). VPS26 appears to assemble and stabilize the complex, while VPS29 acts as a bridge from the core complex to a secondary complex of hetero- og homo-dimers of sorting nexins (SNXs). Based on their Phox homology domain, SNXs interact with phosphatidylinositol-3-phosphate (PI3P) containing membranes (Oliviusson et al., 2006; Zelazny et al., 2013). Notably, the SNX subunit appears to be dispensable for some functions, contrary to the situation in mammals, which may indicate that the plant retromer has evolved separate functions (Pourcher et al., 2010).

Figure 1.6: 3 weeks old Ler wild type plants compared to laz5-D57 acd11, acd11 NahG and acd11 NahG laz4 plants, before and after BTH treatment. Scale bar indicates 1 cm.

Figure 1.7: Schematic overview of the retromer complex.
In plants the retromer is believed to function in retro-transport of cargo from late endosomal compartments to the TGN (Figure 1.8). Knockout of retromer components results in mis-localization of storage proteins originally meant for storage vacuoles, and loss of cellular polarity through mis-localization of auxin transporters to the vacuole (Shimada et al., 2006; Fuji et al., 2007; Jaillais et al., 2007; Yamazaki et al., 2007; Kleine-Vehn et al., 2008). Components of the retromer are relatively well conserved across species, but most of the research in plants has focused on VPS29. There is only one VSP29 homolog, in contrast to VPS26 and VPS35 with two and three homologs respectively (Yamazaki et al., 2007). Sequence analysis of the remaining three members of the *laz4* complementation group did not reveal mutations in any genes of the core retromer complex, indicating that currently unidentified interactors may be involved in *acd11*-related PCD (D. Hofius). One of the most well described cases for retromer deficiency, is for the mis-localization of the vacuolar hydrolase carboxypeptidase Y in yeast that occur when knocking out VPS35, the SNX homolog VPS5 or the VPS10 sorting receptor (Paravicini et al., 1992; Horazdovsky et al., 1997). This is also seen in mammals, where the cation-independent mannose-6-phosphate receptor has the same role as VPS10 and mis-sorts cathepsin D upon knockout, which could mean that there is vacuolar dysfunction in retromer mutants (Arighi et al., 2004). How the retromer complex could be involved in *acd11*-associated PCD will also be discussed in section 3.1 of the results.

![Figure 1.8: Schematic overview of how the retromer complex is involved in retrograde transport from late endosomal compartments to the TGN.](image-url)
2. Aim of this thesis

This thesis is divided into three parts, discussed in sections 3.1, 3.2 and 3.3. Each section will contain a brief introduction to its topic, followed by results and a discussion.

In section 3.1 I will focus on PCD in the *acd11* mutant. I will mention the weak suppression of the *laz4-1* mutant in BTH-triggered *acd11 NahG* PCD, and characterize the role of the three VPS35 homologs in *acd11*-associated and effector-triggered PCD, and discuss the retromers role in virulent and avirulent-triggered immunity. Its connection to autophagy and its position as a regulator downstream of LAZ5 and other potential *R* proteins will also be analyzed. In addition, I will present sphingolipid profiling of *acd11*-related cell death compared to Ler WT, and briefly comment on the function of ACD11 based on structural data. The retromer work has been carried out with Daniel Hofius (Dept. of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences), while sphingolipid profiling and provided structural data on ACD11 was done in collaboration with Rhoderick E. Brown (Hormel Institute, University of Minnesota, USA).

Section 3.2 builds on information obtained on ACD11 in *Arabidopsis* and the work done by Morten Petersen and Søren Skov, who observed that knockdown of *HsfFAPP2* leads to accumulation of autophagosomes in addition to cell death. Here I will analyze the apparent contribution of autophagy upon siRNA knockdown of FAPP2 in mammalian cells. This work was done as a collaboration with Søren Skov (Laboratory of Immunology, U. Copenhagen), and Anne Simonsen under the supervision of Maria Lyngaas Torgersen (Section for Biochemistry, Institute for Basic Medical Sciences, U. Oslo).

Finally, section 3.3 approaches the contrasting observations of autophagy as a pro-life or pro-death pathway during and after effector-triggered PCD. By comparing responses between autophagy-deficient mutants and NPR1 knockouts, the latter which has been proposed by Yoshimoto et al. (2009) to be a direct regulator of autophagy during HR, I will try to distinguish between different roles for the autophagic machinery. Using mutants at various ages, analyses of ubiquitin levels along with ER stress and avirulent infections will be presented to establish a hypothesis that takes the observations presented in the literature into account.
3. Results

3.1 Characterization of the retromer complex in immunity and cell death

In a screen for suppressors of BTH induced immunity and cell death in mutagenized acd11 NahG plants, several different potential mutants were identified (Malinovsky et al., 2010). The identification of the laz5 suppressor along with knockouts of the HR-regulators EDS1 and PAD4, indicated that acd11-related cell death is R protein triggered (Malinovsky et al., 2010; Palma et al., 2010). A relatively weak suppressor identified was that of laz4-1 (Figure 1.6). Map based cloning identified the At1g75850 gene as having a single point mutation in an intron acceptor site, inserting a premature stop codon downstream of the mutation. At1g75850 encodes for VPS35b, one of three homologs (VPS35a, AT2G17790; VPS35c, AT3G51310) present in Arabidopsis that make up part of the retromer, a complex used for recycling cargo between the late endosomal compartments. Complementation of Ler acd11-1 NahG laz4-1 with a genomic construct of VPS35b restored the BTH induced PCD phenotype to that observed for acd11 NahG, verifying that the mutation in At1g75850 was the reason for the PCD suppression. Further analyses of T-DNA knockouts of all three VPS35 homologs (vps35a-1, vps35b-1, vps35c-1; Yamazaki et al. (2007) [Note that the VPS35a and VPS35b designations in Yamazaki et al. (2007) are swapped compared to the TAIR annotations. This thesis uses the TAIR nomenclature]) did not indicate any suppression of the acd11 phenotype in both Ler and Col-0 ecotypes. Effector triggered immunity by Pst DC3000 avrRpm1 was also not affected in any of the mentioned single vps35 mutants.

3.1.1 vps35 double knockout mutants suppress acd11-associated cell death

Redundancy between VPS35 homologs have been observed before, so previously published vps35b vps35c and vps35a vps35c double mutants were acquired (Yamazaki et al., 2007), while vps35a vps35b was generated. While single vps35 and vps35b vps35c mutants don’t display any phenotypic differences from WT, vps35a vps35b and vps35a vps35c have a semi-dwarf growth phenotype (Figure 3.1.1; Supplemental figure 7.1). The growth
dependent phenotype may be dependent on hormone signaling, as auxin transporters are dependent on SNX1 and VPS29 for correct sorting, and thus most likely also dependent on other retromer components such as VPS35 (Jaillais et al., 2006; Kleine-Vehn et al., 2008a). *acd11* was introduced into the three *vps35* double mutants, and surprisingly suppression of PCD was observed for the *vps35a vps35b acd11* and *vps35b vps35c acd11* triple mutants. Although the suppression did not fully rescue the PCD phenotype under short day conditions (8 hours of light) at 21°C, these mutants were capable of surviving and set viable seeds if they were kept under close care, contrary to *acd11*. These observations again indicates functional redundancy between VPS35 members, but also that VPS35b of the retromer complex is involved in *acd11*-associated cell death regulation. Interestingly, the *vps35a vps35c acd11* triple mutant was observed to experience enhanced PCD development, as it died even faster than *acd11* (Figure 3.1.1). A possible explanation for this behavior will be discussed later in this section. Notably, when grown under 17°C short day conditions, suppression of PCD was even more pronounced, as the cell death phenotype was even further suppressed, indicating that endosomal trafficking is the reason for suppression (Figure 3.1.2; Kuismanen et al., 1989). These findings indicate a role for VPS35 in the genetic regulation of *acd11*-associated cell death.

**Figure 3.1.1:** Photos of 4 weeks old Col-0 WT and *acd11* plants grown under short day conditions at 21°C, compared to double *vps35* knockout mutants crossed to *acd11*. Scale bar indicates 1 cm.
3.1.2 VPS35 is involved in LAZ5-independent cell death

Since \textit{vps35a vps35b} and \textit{vps35b vps35c} double mutants appear to suppress the \textit{acd11} PCD phenotype, it is possible that VPS35 is specifically involved in regulation of LAZ5, or that it is a general regulator of \textit{R} proteins or PCD pathways during HR. Under short day growth conditions, the presence of a T-DNA insert in LAZ5 (\textit{laz5-1}) appear to completely restore the PCD phenotype in Col-0 \textit{acd11} (Palma \textit{et al.,} 2010). If transferred to long day conditions (16 hours of light), the Col-0 double mutant develop a secondary PCD effect, which could potentially be the activation of a secondary \textit{R} protein (Figure 3.1.3A). RIN4 is guarded by the two \textit{R} proteins RPS2 and RPM1, so it is possible that a similar guard arrangement is the case for ACD11 (Axtell \textit{et al.,} 2003; Mackey \textit{et al.,} 2002). To evaluate if VPS35b is involved solely in the regulation of LAZ5 triggered PCD or if it also contributes to additional PCD pathways, the single \textit{vps35b} knockout was introduced into Col-0 \textit{laz5 acd11}. As seen in figure 3.1.3B, the \textit{laz5 vps35b acd11} triple mutant show much improved

\textbf{Figure 3.1.2}: Photos of 4 weeks old Col-0 WT and \textit{acd11} plants grown under short day conditions at 17°C, compared to crosses between double \textit{vps35} knockout mutants and \textit{acd11}. Scale bar indicates 1 cm.
growth under long day conditions compared to laz5 acd11. While the secondary PCD phenotype was not completely suppressed, it indicates that VPS35b is involved in LAZ5-independent cell death regulation.

3.1.3 Effector-triggered PCD is partially dependent on VPS35

To evaluate the contribution of VPS35 in effector triggered PCD, Pst DC3000 carrying the avrRpm1 effector was injected into the double vps35 mutants and cell death was quantified using an ion leakage assay that measure the concentration of ions released from infected leaf discs during PCD development (Mackey et al., 2003). AvrRpm1 specifically targets the defense regulator RIN4 and hyper-phosphorylates it (Mackey et al., 2002). The R protein RPM1 detects this modification, and triggers a strong PCD response through multiple parallel pathways that involves both autophagy, cathepsins, metacaspases and proteasomal-dependent vacuolar collapse (Mackey et al., 2002; Hofius et al., 2009; Coll et al., 2010; Hatsugai et al., 2009; McLellan et al., 2009; Gilroy et al., 2007). This makes avrRpm1 triggered PCD an excellent tool for testing the general contribution of VPS35 to PCD during HR. As seen in figure 3.1.4, the vps35a vps35b double mutant did not show any
significant difference compared to WT. Strikingly, \textit{vps35a vps35c}, and particularly the \textit{vps35b vps35c} double mutant, were observed to show significant difference in ion leakage. First, these results imply that VPS35 is involved in the regulation of \( \text{R} \) protein triggered cell death downstream of \( \text{R} \) proteins other than LAZ5. Second, this suggests that VPS35b is not the primary homolog in RPM1-triggered PCD, indicating that the different homologs may support different roles during the HR-associated cell death.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.1.4.png}
\caption{Ion leakage in 5 weeks old \textit{vps35} double mutants after infection with \textit{Pst DC3000 avrRpm1}. Col-0 WT and \textit{rpm1} serves as controls. Mean and standard deviations were calculated from 6 discs per treatment with 4 replicates. Pairwise comparisons at the last time point post infection for means versus WT were performed using one-way ANOVA test followed with Dunnett post-hoc test. ns, not significant; *, \( P \leq 0.05 \); ****, \( P \leq 0.0001 \).
}
\end{figure}

\textit{RPS2}, the other CC-NB-LRR that also guards RIN4, was triggered by injection with \textit{Pst DC3000 avrRpt2}. While the kinetics of the assay were observed to be different to that of \textit{avrRpm1}, the outcome was similar in that \textit{vps35a vps35b} was not significantly different from WT and \textit{vps35a vps35c} and \textit{vps35b vps35c} were (Figure 3.1.15). Resembling results were also obtained with cell death triggered by \textit{RPS5}, a CC-NB-LRR \( \text{R} \) protein that detects the degradation of PBS1 by the \textit{Pst DC3000} effector \textit{avrPphB} (Data not shown; Shao \textit{et al.}, 2003).
Similarly to avrRpt2, the *Pst* DC3000 effector avrRps4 induce a slower and more prolonged HR than that of avrRpm1, as measured by ion leakage. They also differ in the downstream regulatory components, as the TIR-NB-LRR RPS4 triggers EDS1-dependent autophagy in the presence of avrRps4, which RPS2 does not do upon detection of avrRpt2-mediated degradation of RIN4 (Hinsch et al., 1996; Hofius et al., 2009; Aarts et al., 1998; Wirthmueller et al., 2007; Mackey et al., 2003). Upon *Pst* DC3000 avrRps4 infection, both the *vps35a vps35b* and *vps35b vps35c* were not significantly different from WT (Figure 3.1.6).

Consistent with the results observed for avrRpm1 and avrRpt2, the *vps35a vps35c* double mutant displayed very strong suppression of cell death. It is surprising to see that the published *rps4* knockout allele was also not significantly different from WT, which could hypothetically mean that more than one *R* protein is involved in the recognition of the avrRps4 effector (Wirthmueller et al., 2007). RPS4 interacts directly with EDS1 and avrRps4 disrupts this complex, allowing RPS4 to relocalize and activate defense signaling (Bhattacharjee et al., 2011; Heidrich et al., 2011).

**Figure 3.1.5:** Ion leakage in 5 weeks old *vps35* double mutants after infection with *Pst* DC3000 avrRpt2. Col-0 WT and *rps2* serves as controls. Mean and standard deviations were calculated from six discs per treatment with 3 replicates. Pairwise comparisons at the last time point post infection for means versus WT were performed using one-way ANOVA test followed with Dunnett post-hoc test. ns, not significant; *, *P* ≤ 0.05; ****, *P* ≤ 0.0001.
Since VPS35 appears to be involved in RPS2 and RPM1-triggered cell death, it is possible that introduction of knockout alleles of \textit{vps35} into the \textit{rin4} LMM could potentially rescue the seedling lethal mutant. Unfortunately, it was not possible to obtain a triple mutant, even after screening more than 50 plants each of \textit{rin4} crossed to the double \textit{vps35} mutants (Data not shown). Instead, to test the possible suppression of LMMs, the double \textit{vps35} mutants were crossed to the conditional LMM \textit{lsd1} (Dietrich \textit{et al.}, 1994). As shown in figure 3.1.7, the \textit{vps35a vps35c lsd1} triple mutant displayed an enhanced phenotype, compared to both \textit{lsd1} single mutants, but also to \textit{vps35a vps35c} double mutants (Latter not shown). This observation is similar to what was observed when crossing \textit{vps35a vps35c} into \textit{acd11} (Figure 3.1.1). Upon repeated BTH treatment, a normal stimuli for induction of the runaway cell death phenotype of \textit{lsd1}, no apparent suppression was observed for \textit{vps35a vps35b lsd1} and \textit{vps35a vps35c lsd1}. In contrast, the \textit{vps35b vps35c lsd1} triple mutant almost completely suppressed the BTH-induced cell death, compared to the \textit{lsd1} control.
Considering all of these observations, it strongly indicates that immunity-associated regulation of cell death requires contribution of VPS35.

3.1.5 VPS35 is required for resistance against *Pst* DC3000 avrRps4

PCD during HR is considered to be required for full resistance against biotrophic pathogens, although there are examples of mutants where this is not the case, such as with the DND mutants (Clough *et al*., 2000; Jurkowski *et al*., 2004; Yu *et al*., 1998). To elucidate if basal resistance to virulent *Pst* DC3000 was compromised, bacteria were injected and assayed for growth in the *vps35* double mutants. Bacterial growth in the *vps35* double mutants were comparable to that of WT, in contrast to the *eds1* mutant, in which ten times higher bacterial growth was observed 3 days after infection, illustrating that VPS35 is not involved in basal resistance to *Pst* DC3000 (Figure 3.1.8A). To see if the observed suppression of effector-triggered PCD affected resistance, *Pst* DC3000 carrying avrRpm1, avrRpt2 or avrRps4 was infiltrated. Similar to the tendency observed for virulent *Pst* DC3000, RPM1 and RPS2-activated resistance was unaffected for all *vps35* double mutants, compared to WT (Figure 3.1.8B and 3.1.8C). While CC-NB-LRR-associated resistance appear unaffected, RPS4-triggered PCD appeared to significantly compromise resistance in the *vps35a vps35c* double mutant (Figure 3.1.8D). In all effector triggered ion leakage

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assays this particular mutant has been observed to consistently be the strongest suppressor, arguing for a role of VPS35a and VPS35c in TIR-NB-LRR-triggered resistance.

Taken together, all of these results provide evidence that VPS35 is involved in the genetic regulation of PCD during HR, in particular disease resistance downstream of the TIR-NB-LRR type R protein.

Figure 3.1.8: Bacterial growth of *Pst* DC3000 carrying (A) none, (B) avrRpm1, (C) avrRpt2 or (D) avrRps4, 0 and 3 days after injection, in WT and vps35 double mutants. *eds1* (A + D), *rpm1* (B) and *ndr1* (C) used as controls for increased susceptibility. Values are log10 transformed, plus/minus standard deviation calculated from 3 (A, B) or 4-6 (C, D) leaf discs per treatment with 3 or 4 replicates. Pairwise comparisons 3 days post infection for means versus WT were performed using one-way ANOVA test followed by Tukeys post-hoc test. ***, P ≤ 0.001.
3.1.6 Cell death regulation by VPS35 is also dependent on VPS29/MAG1

In plants the retromer is believed to be a multimeric complex comprised of VPS35, VPS26 and VPS29/MAG1 (Oliviusson et al., 2006; Zelazny et al., 2013). *Arabidopsis thaliana* has only a single homolog of VPS29/MAG1, which is believed to be required for membrane interactions and interactions with SNX dimers (Yamazaki et al., 2007; Oliviusson et al., 2006). To establish if the results obtained for the *vps35* double mutants were mimicked for other components of the retromer complex, a leaky allele of VPS29/MAG1 (*mag1*) was acquired and assayed for contribution to effector-triggered cell death, as measured by ion leakage (Shimada et al., 2006; Yamazaki et al., 2007). In accordance with the results observed for the *vps35a vps35c* double mutant, ion leakage in the *mag1* mutant was significantly different from WT when infected with *Pst* DC3000 carrying avrRpt2 and avrRps4 (Figure 3.1.9 and 3.1.10). Similarly, resistance was compromised for the *mag1* mutant when bacterial growth was assessed for bacteria expressing avrRps4, but not for avrRpt2 (Figure 3.1.11A and 3.1.11B). Overall these results demonstrate a role for the entire retromer complex in HR PCD regulation, and partially in *R* protein triggered resistance.

![Figure 3.1.9: Ion leakage in 5 weeks old *mag1* after infection with *Pst* DC3000 avrRps4. Col-0 WT, *vps35a vps35c* and *rps4* serves as controls. Mean and standard deviations were calculated from six discs per treatment with 3 replicates. Pairwise comparisons at the last time point post infection for means versus WT were performed using one-way ANOVA test followed with Dunnett post-hoc test. ns, not significant; **, P ≤ 0.01; ****, P ≤ 0.0001.](image)

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Figure 3.1.10: Ion leakage in 5 weeks old mag1 after infection with Pst DC3000 avrRpt2. Col-0 WT, vps35a vps35c and rps2 serves as controls. Mean and standard deviations were calculated from six discs per treatment with 3 replicates. Pairwise comparisons at the last time point post infection for means versus WT were performed using one-way ANOVA test followed with Dunnett post-hoc test. **, P ≤ 0.01; ****, P ≤ 0.0001.

Figure 3.1.11: Bacterial growth of Pst DC3000 carrying (A) avrRps4 or (B) avrRpt2, 0 and 3 days after injection, in WT and mag1 mutants. eds1 (A) and rps2 (B) used as controls for increased susceptibility. Values are log10 transformed, plus/minus standard deviation calculated from 3 leaf discs per treatment with 4 replicates. Pairwise comparisons 3 days post infection for means versus WT were performed using one-way ANOVA test followed by Tukeys post-hoc test. ****, P ≤ 0.0001.
3.1.7 Autophagy appear to be compromised in retromer mutants

The vps35a vps35c double mutant, and the mag1 mutant both show signs of early senescence, reminiscent of mutants that are deficient in autophagy, such as atg7 or atg2 (Supplemental figure 7.1; Doelling et al., 2002). Previously it was shown in our group that PCD during HR is dependent on autophagy by a subset of R proteins, including RPM1 and RPS4 (Hofius et al., 2009). This could indicate that autophagy-deficiency is a cause for the suppression observed during effector triggered ion leakage. To verify that the vps35 and mag1 mutants were autophagy deficient, leafs were excised and kept in the dark for 4 days (Figure 3.1.12). Leafs from vps35a vps35b and vps35b vps35c both retained their green phenotype, indicating that they are not autophagic deficient, correlating with the relatively weak or no suppression observed during ion leakage assays. In contrast, similarly to the atg7 and atg2 mutants, vps35a vps35c and mag1 both displayed early senescence in the excised leafs, probably due to lack of the machinery required for recycling nutrients.

![Figure 3.1.12](image-url): Photos of detached leafs from 4 weeks old WT, atg7, atg2, mag1 and vps35 double mutant plants. Leafs were excised and kept for 4 days in the dark under high humidity.

Selective degradation of cargo by autophagy is dependent on cargo adapters, such as NBR1 in Arabidopsis, for targeting substrates to the autophagosomes. Accumulation of NBR1 is observed in autophagy-deficient mutants, since maturation or degradation of the autophagosome is blocked, preventing degradation of the adapter (Figure 3.1.13; Svenning et al., 2011). Additional confirmation of autophagy-deficiency in the vps35a vps35c and mag1 backgrounds, is provided by Daniel Hofius. Strong accumulation of NBR1 is observed in atg2 compared to WT and vps35b vps35c (Figure 3.1.13). This reveals that there
presumable is no autophagic deficiency during development in that particular \textit{vps35} mutant, as NBR1 is tuned over in the same way as in the WT. On the other hand, protein levels of NBR1 is higher in the \textit{vps35a vps35b, vps35a vps35c} and \textit{mag1} mutants, partially mimicking the results observed for the detached leaf assay, and indicating that the autophagic machinery required for degradation is dysfunctional (Figure 3.1.12 and 3.1.13). After infiltration with \textit{Pst} DC3000 avrRpm1, NBR1 degradation seem to follow a biphasic response, with protein accumulation 2 and 6 hours after infiltration in WT (Figure 3.1.14). While this is also clearly observed for the \textit{vps35b vps35c} mutant, \textit{vps35a vps35b, vps35a vps35c} and \textit{mag1} all lack the degradation of NBR1 4 hours after infiltration, instead accumulating NBR1 to an even higher extent. The lack of a biphasic response of NBR1 degradation is indicative of a role for the retromer complex during NBR1-dependent autophagic PCD, and can in part explain the observed suppression of PCD observed in the ion leakage assays.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{immunoblot.png}
\caption{Immunoblot detection of NBR1 in 10 days old seedlings of Col-0 WT and \textit{atg2} controls in comparison to \textit{mag1} and double \textit{vps35} mutants. The large subunit of rubisco serve as loading control. Data provided by Qinsong Liu from Daniel Hofius’ group.}
\end{figure}
3.1.8 Sphingolipid metabolism during acd11-associated cell death

Lack of autophagic PCD during HR can potentially explain the results obtained for the ion leakage after infection with Pst DC3000 carrying avrRpm1 and avrRps4, but not those for avrRpt2, as it does not trigger autophagy in a pro-death manner during HR (Hofius et al., 2009). Instead other PCD pathways may be involved during HR. laz4 and in turn VPS35b was identified in a screen for suppressors of acd11-associated cell death, which could indicate a role of sphingolipid metabolism in LAZ5-triggered PCD (Liang et al., 2003; Cuvillier et al., 1996; Gomez-Munoz et al., 1997; Peer et al., 2010; Pata et al., 2010). ACD11 was originally characterized as being able to catalyze the transport of sphingosine between membranes in vitro, and was later found to be able to do the same with sphingomyelin, although the latter is not present in plants (Brodersen et al., 2002; Petersen et al., 2008). Through structural homology modeling and structure determination at 1.8Å after crystallization of ACD11, Rhoderick Browns group found that ACD11 share a characteristic GLTP fold, similar to that found for HsGLTP (Simanshu et al., 2014). Structural features indicated a more basic binding pocket in ACD11 compared to HsGLTP,
which could explain the lack of glycolipid transfer (Simanshu et al., 2014, Petersen et al., 2008). Förster resonance energy transfer analyses with potential substrate lipids identified high transfer rates of C1P and phyto-C1P by ACD11, but not of closely related sphingolipid species. Competition assays and analysis of a crystal structure of ACD11 with bound C1P (Albeit with a single amino acid substitution due to stability issues) confirmed close interaction between the sphingolipid and the binding pocket, confirming ACD11 as a C1P transfer protein (Simanshu et al., 2014).

Since ACD11 is guarded and a regulator of sphingolipid metabolism, it is reasonable to assume that its removal would affect sphingolipid metabolism in a negative manner for the host during pathogen defense (Heung et al., 2006). In apparent confirmation of this, Ler acd11-1 also accumulate sphingolipid species to levels higher than Ler WT (Simanshu et al., 2014). The confirmation of C1P transfer activity by ACD11 and the fact that the ceramide kinase acd5 is also a LMM, can indicate that are central components during resistance (Greenberg et al., 2000; Liang et al., 2003). In addition, the balance between the levels of ceramide and C1P is considered important for the choice between life and death (Liang et al., 2003; Cuvillier et al., 1996; Gomez-Munoz et al., 1997). To evaluate the changes in sphingolipid levels during acd11-associated cell death, and not growth-related perturbations, Ler acd11-1 NahG plants were treated with BTH and analyzed. Seventy-two and 120 hours after treatment, it was found that ceramide levels rose dramatically compared to WT and NahG, along with a minor increase in 2-hydroxyceramide, which may be due to increased ceramide turnover. In comparison, levels of C1P were not detectable (Simanshu et al., 2014). By treating plants with cold temperatures, it is possible to induce a rapid and significant increase of C1P and phosphorylated LCBs, mediated by nitric oxide (Cantrel et al., 2010). So to analyze C1P species in acd11, Ler NahG, acd11-1 and acd11-1 NahG plants were treated at 4°C for 4 hours prior to BTH treatment. Figure 3.1.15 show that C1P species are now detectable after cold treatment. BTH treatment of NahG, compared to untreated NahG, show that SA responses in itself does not appear to trigger fluctuations of sphingolipid species, except for C1P having 20:0 carbons long acyl chains. In stark contrast, several species of C1P were significantly up-regulated in acd11 along with BTH treated acd11 NahG, up to five fold
compared to *NahG*. This surge in C1P species indicates that loss of *acd11* is triggering an increase either due to lack of ACD11-mediated transport activity, or due to indirect cell death activity. Significant up-regulation was also observed for untreated *acd11 NahG*, which indicate that functional loss of ACD11 is the direct reason for the accumulating C1P species (Figure 3.1.15). While there was no statistical significance between treated and untreated *acd11 NahG* (Statistical indicators not shown), there was a slight trend for higher levels of all species in the treated samples, suggesting that a minor increase of C1P species may occur during HR-triggered PCD.

Sample deviation is high in several of the samples, which may be the cause for lack of statistical significance for several C1P side chains when comparing *NahG* to other genotypes. The BTH treatment time point was selected based on the observation that ceramide species in general were significantly up-regulated after 72 hours in BTH treated *acd11 NahG* (Simanshu *et al.*, 2014). A higher sample size may provide a better picture, but it is also possible that the time frame of the cold or BTH treatments are too short for sphingolipid metabolism to stabilize.
Cold treated samples were also analyzed for presence of phyto-C1P, as ACD11 appear to transport this similar sphingolipid. In contrast to the data observed for C1P, BTH treatment of NahG induced phyto-C1P perturbations for the majority of acyl chain lengths (Figure 3.1.16). It shows that the two sphingolipids have different roles in regulation of BTH-triggered responses, meaning that phyto-C1P and not C1P may be involved SAR regulation (Lawton et al., 1996). That BTH has an effect by itself on this sphingolipid species may make it harder to determine the exact contribution of ACD11 transport function of phyto-C1P levels. Phyto-C1P levels in acd11 were significantly higher than both treated and untreated NahG for all side chain lengths, indicating that prolonged cell death induces further sphingolipid changes (Supplemental table 1). Whether other GLTP proteins in Arabidopsis can transfer phyto-C1P is not known. Interestingly, BTH treated acd11 NahG was also significantly higher for the majority of species compared to BTH treated NahG, indicating that BTH may amplify the sphingolipid response from loss of ACD11 function.

Figure 3.1.15: Profile of C1P species in 5 weeks old Ler NahG, acd11 and acd11 NahG plants treated or untreated with BTH. All samples were cold shocked for 4 hours at 4°C prior to the timepoint where selected sampled were treated with BTH. Values are plus/minus standard deviation calculated from 3 replicates. Pairwise comparisons means versus NahG were performed using student t-test. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.
The suppression by vps35 double mutants in acd11 could potentially rely on PCD pathways that are triggered by changes in sphingolipid metabolism. Endosomal membranes contain sphingolipids, and the retromer complex may be dependent on specific species for correct functionality (Markham et al., 2007; Markham et al., 2013). Blocking ACD11-dependent C1P transport may lead to the accumulation of specific C1P precursors, such as LCBs, that have been observed to induce PCD through ROS-dependent signaling (Shi et al., 2007; Kuroyanagi et al., 2005). It is possible to induce changes in sphingoid species and raise levels of free sphingoid bases, by treating with the sphingoid-like mycotoxin FB1, an inhibitor of the ceramide synthase SPT (Abbas et al., 1994; Peer et al., 2010; Shi et al., 2007). As seen in figure 3.1.17, FB1 induced lesions in WT leafs after 7 days. No effect of the toxin was observed in NahG, as FB1-induced cell death is dependent on SA and the action of VPEs (Asai et al., 2000; Kuroyanagi et al., 2005). Similar to WT, all four retromer knockout mutants developed clear spread of lesions, suggesting that the
retromer is not involved in sphingolipid-dependent signaling, nor in the transport or activity of VPEs (Figure 3.1.17).

![Figure 3.1.17: Photos of detached leaves from 4 weeks old WT, NahG, mag1 and vps35 double mutant plants grown under long day conditions, 7 days after injection with 10 μM FB₁ or 0.5% methanol (Mock).]

### 3.1.9 Discussion of retromer-dependent regulation of HR-associated PCD

*laz4* was initially identified in a screen for suppressors of *acd11*-associated cell death and found to encode for VPS35b, a member of the retromer complex. This multimeric complex binds to the external surface of endosomal compartments and is composed of a cargo recognition sub complex consisting of VPS26-VPS35-VPS29 and a membrane interacting SNX dimer (Attar *et al.*, 2009). It is believed to bind directly to membrane embedded cargo adapters that mediate interaction with cargo needed to be recycled from late to early endosomal compartments. The core VPS components are well conserved across eukaryotic species, while homologs of SNXs are more variable, probably representing specialization or retromer independent functions (Carlton *et al.*, 2005; Haft *et al.*, 2000; Edgar *et al.*, 2000; Attar *et al.*, 2010). Similar to the functionality in yeast and mammals, the plant retromer
interacts with sorting receptors, and recycle them from the late endosomal compartment to
the TGN (Niemes et al., 2010; Kang et al., 2012; Oliviusson et al., 2006; Seaman et al., 1998).
Genetic analyses have revealed multiple developmental phenotypes upon loss of retromer
components, such as misrouting of storage protein out of cells, mis-localization of auxin
transporters, loss cell polarity and improper organ development (Shimada et al., 2006;
Yamazaki et al., 2007; Jaillais et al., 2006; Kleine-Vehn et al., 2008a, Jaillais et al., 2007).

In this thesis I identify a novel role for the retromer complex in immunity-
associated cell death regulation. Introduction of vps35 knockouts in acd11 identified
redundancy in cell death regulation, and also a specific dependence on the VPS35b
homolog (Figure 3.1.1 and 3.1.2). Further analyses indicated that this cell death regulation
may be LAZ5-independent, suggesting that more than one R protein are activated upon
ACD11 removal (Figure 3.1.3). The reason for the relatively weak suppression of the vps35
double in acd11-dependent cell death phenotype may be due to suppression of only one of
two active R proteins (Figure 3.1.1 and 3.1.2). LAZ5 is a TIR type NB-LRR similar to RPS4
and share similar downstream components (Palma et al., 2010). All R proteins appear to be
dependent on EDS1, supposedly through SA amplification, and in the case of RPS4, also
through direct interaction with EDS1 (Wiermer et al., 2005; Bhattacharjee et al., 2011;
Heidrich et al., 2011). In contrast, R proteins of the CC-NB-LRR type appear to not be
regulated directly through EDS1, but instead through NDR1, relying on EDS1 only for SA
amplification (Wiermer et al., 2005; Aarts et al., 1998; Hofius et al., 2009). Since vps35b
suppress the late stage onset of PCD in laz5 acd11 under long day conditions, it may
indicate that VPS35 act through NDR1-dependent signaling pathways. As seen in figure
3.1.7, acd11 is not the only LMM that may depend on CC-NB-LRR triggered PCD regulated
by VPS35, as the introduction of vps35b vps35c also suppressed BTH-induced cell death in
the lsd1 mutant (Bonardi et al., 2011). This also strongly suggests that VPS35 may support
PCD development downstream of more than one R protein. Furthermore, this is
supported by the observation that cell death triggered by the CC-NB-LRR R proteins RPS2
and RPM1 are also suppressed by both vps35a vps35c and vps35b vps35c (Figure 3.1.4,
3.1.5). In support of the NDR1-dependent theory, introduction of a loss-of-function allele
of NDR1 appear to fully rescue the late cell death phenotype observed for laz5 acd11 under
long day conditions (C. Greeff, personal communication). NDR1 is associated with RIN4 at
the plasma membrane, and appear to be involved in membrane-cell wall adhesion, giving
a potential clue how a second R protein guarding ACD11 could trigger PCD (Knepper et
al., 2011; Day et al., 2006). Interestingly, RPS4-triggered cell death is also suppressed by the
vps35a vps35c double mutant, suggesting that VPS35 is involved in a more general way of
regulating cell death downstream of R proteins, and not just downstream of CC-NB-LRRs
(Figure 3.1.6). It is possible that specific R proteins regularly move through endosomal
compartments, and if not recycled back to the TGN by the retromer, become degraded in
the vacuole. Analyses of R protein levels in retromer mutant backgrounds would provide
insight into this hypothesis, and is currently being investigated in Daniel Hofius’ group.

Effector-triggered PCD during biotrophic infections is generally considered to be
crucial for establishing a defense response that is strong enough to deter pathogen growth
(Grant et al., 1995; Century et al., 1995). Therefore it is surprising to see that the highly
significant cell death suppression observed in the ion leakage assays did not have an effect
on resistance against avirulent strains of Pst DC3000 carrying avrRpt2 and avrRpm1
(Figure 3.1.8B, 3.1.8C and 3.1.11B). DND mutants display no cell death induction, but still
retain high levels of PR gene expression, suggesting that an active immune response is
occurring continuously (Yu et al., 1998). It is therefore possible that VPS35 knockout would
have no effect in dnd1 and dnd2 backgrounds. Resistance to virulent Pst DC3000 was also
not compromised in vps35 double knockout backgrounds, indicating that basal resistance
is also not affected. In mammalian species SNXs are involved in the recycling of plasma
membrane receptors, but even with the large number of receptor-like kinases in
Arabidopsis, it appears that VPS35 is not involved in their recycling. It is worth mentioning
that resistance to Pst DC3000 avrRps4 was observed to be significantly different in
bacterial growth assays compared to WT, for both mag1 and vps35a vps35c, although not
susceptible to the extent of the eds1 control (Figure 3.1.8D and 3.1.11A). Since various types
of R genes are regulated differently, the downstream cell death pathways may also
contribute differently to resistance (Hofius et al., 2009). Pst DC3000 is a hemi-biotroph and
that could also explain why cell death mis-regulation does not have an affect. Although
this strain is used extensively in many groups for analyzing avirulent pathogen-plant

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interactions, its hemi-biotrophic nature can cause it to survive under long term cell death responses. Since I assay for growth over 3 days, the pathogen might acclimatize and shift to necrotrophic growth in the dead tissue, rendering cell death defense responses virtually useless. Virulent and avirulent infection assays with obligate biotrophs such as Hpa should therefore be tested in the same retromer knockout backgrounds.

Analyses of effector triggered cell death by avrRpt2 and avrRps4 in a leaky allele of VPS29/MAG1 proved that cell death regulation does not rely solely on VPS35, but appear to be dependent on the entire retromer complex (Figure 3.1.9 and 3.1.10). Suppression was not as extreme as that observed for vps35a vps35c, indicating that the leaky nature of the allele proves that some functionality is still maintained. We have not been able to obtain a full triple vps35a vps35b vps35c knockout and neither has Yamazaki et al. (2007), which imply that VPS35 is required either for embryo or seedling development, or for the events leading to fertilization. A stronger knockout allele of VPS29/MAG1 is available, and it can be that suppression of HR-related PCD is more efficient in this mutant (Shimada et al., 2006). The question is if it makes sense to use this knockout instead of the allele used in this thesis, as a true VPS29/MAG1 knockout also appear to be seedling lethal in Arabidopsis when combined with SNX knockouts (Pourcher et al., 2010). Presently it is unknown if either of the two VPS26 homologs in Arabidopsis are also involved in cell death regulation, but the conserved nature of the retromer suggests that this can indeed be the case (Yamazaki et al., 2007). Curiously, VPS26 and SNX1/SNX2 function appear to be critical for embryo development in mice, suggesting that a retromer null mutant can also not be obtained in plants (Schwarz:2002ej, Lee:1992uz). This notion suggest that R protein triggered cell death could be suppressed even further in a ‘true’ retromer knockout.

One of the downstream cell death pathways that are triggered specifically by avrRps4 and avrRpm1, but not by avrRpt2, is that of autophagy (Hofius et al., 2009). vps35a vps35c and mag1 both display early senescence, which is supported by an accumulation of the autophagic cargo-adapter NBR1 (Figure 3.1.12 and 3.1.13). Infection with Pst DC3000 avrRpm1 appear to trigger a biphasic response, and contrary to WT, vps35a vps35b, vps35a vps35c and mag1 does not have the intermediate degradation of NBR1 (Figure 3.1.14). This strongly indicate that autophagy is dysfunctional in retromer backgrounds.
The retromer complex is known to be required for the recycling of a subset of lysosomal/vacuolar hydrolases dependent on mannose 6-phosphate receptor (M6PR) in mammals or Vps10 in yeast (Haft et al., 2000; Seaman et al., 1998; van Weering et al., 2010; Paravicini et al., 1992; Seaman et al., 1997; Horazdovsky et al., 1997; Arighi et al., 2004; Rojas et al., 2008). As autophagy relies on the vacuole or lysosome for degradation of its contents, lack of vacuolar proteases would induce a block in the autophagic flux. Since the retromer also interacts with VSRs in plants, mis-localization of vacuolar hydrolases is a strong possibility, especially considering the exocytosis of storage proteins targeted for the vacuole observed in retromer knockout mutants (Oliviusson et al., 2006; Shimada et al., 2006; Shimada et al., 2003; Yamazaki et al., 2007). Curiously, VPEs do not appear to function in autophagosome degradation, as all retromer doubles appear to initiate FB1-induced cell death correctly, which is also a good indication that these vacuolar proteases are not localized in the wrong place (Figure 3.1.17; Kuroyanagi et al., 2005). Whether this means that the retromer is not involved in their transport, or if their function is dispensable for autophagosome degradation, needs further investigation.

Morphology of pre-vacuolar compartments is affected in vps35a and mag1, suggesting that general traffic to the vacuole is affected, although this may only be for a subset of substrates destined for the vacuole (Nodzynski et al., 2013). Similarly, the yeast SNX1 homolog VPS5 appears to result in a fragmentation of the vacuole when knocked out, which implies that a more general shuffling of endosomal membrane is happening (Horazdovsky et al., 1997). Whether the SNX homologs present in plants are also required for autophagosome interaction has to be shown, but their role as membrane shaping entities through their BAR domain suggest a role in membrane modulation. Considering that SNX1 is one out of 3 homologs in Arabidopsis, compared to more than 30 in mammals, it should be much easier to look for lack of autophagosome formation in knockout backgrounds (McGough et al., 2011; Jaillais et al., 2006). Several other observations from other species suggest that autophagic dysfunction is a general phenomenon in retromer deficient backgrounds (Dengjel et al., 2012). One of the major regulators of autophagy in yeast, VPS34, part of the PI3K complex, directly regulate retromer function through the PX domains of SNXs (Burda et al., 2002; Carlton et al., 2004). This indicate that the retromer is
targeted directly to autophagosomes, and this also appears to be the case in yeast (Dengjel et al., 2012). An indirect observation from humans confirms this association, as VPS29 interacts with a Rab GTPase activating protein (GAP), and surprisingly this binding is affected by ATG8, suggesting a switch for the between endosomal membranes and the autophagosome (Popovic et al., 2012). At least one Rab GTPase has been shown to regulate autophagy in Arabidopsis, implying that similar features for retromer-autophagosome association can exist in plants (Kwon et al., 2013). Similar to the observations shown for the retromer in this thesis, this endosomal regulator is involved in regulation of senescence and is also unaffected in FB1-induced cell death execution (Kwon et al., 2009). Since most components move through the endosomal departments an into the vacuole to be degraded, it raises a question of why the retromer would need to target things from the late endosomal compartments to the autophagosome. One possible explanation is that the retromer might function in transport from non-canonical locations, which has been observed on at least one occasion where the retromer was found to move cargo from the Golgi to the ER. This is intriguing considering that isolation membrane may start its formation at ER-mitochondrial contact sites (Hamasaki et al., 2013; Rowland et al., 2012; Ge et al., 2013). If the retromer targets the localization of autophagosome formation, another possible way that autophagy could be blocked is through a shortage of membrane material. VTI11 is a TGN and vacuolar localized Q-SNARE involved in fusion of endosomal membranes and surprisingly, introduction of a vps35a knockout partially suppress the abnormal morphology phenotypes of vti11 (Hashiguchi:2010kp). Intriguingly, a close and partially redundant homolog of VTI11, VTI12, show early senescence when knocked out, similar to autophagy-deficient mutants, suggesting that it is involved in membrane fusion required for autophagosome formation (Surpin et al., 2003). Better insight into the localization of the retromer during autophagy-inducing conditions could shed more light on this hypothesis.

Why vps35a vps35b does not show the early senescence phenotype, but still appear to have trouble with degradation of NBR1 is still unknown (Figure 3.1.12 and 3.1.13). One possibility is that it is not the entire autophagic pathway that is non functional and that only certain cargos are being affected. NBR1 has previously been shown to be required for
abiotic stress responses, but dispensable for both biotic stressors and induced senescence (Zhou et al., 2013). This could prove that \textit{vps35a vps35b} is specifically mis-regulating the targeting of NBR1 to the autophagosome, but not affecting other potential autophagic cargo adapters. Co-localization or interaction studies between NBR1 and retromer components would be indicative if such a function is possible.

Since autophagy appears to be dysfunctional in the \textit{vps35a vps35c} background, it may well explain why it does not suppress \textit{acd11-} and \textit{lsd1-}associated cell death (Figure 3.1.1 and 3.1.7). Even though the autophagic machinery is not capable of working as a pro-death pathway, it is also not capable of responding as a pro-life pathway in \textit{vps35a vps35c} and \textit{mag1}. Autophagy is a general response to cellular stress, and vital for maintaining homeostasis, so when an immune response is triggered it is followed by a sustained stress that can not be dealt with (Kaushik et al., 2010; Lu et al., 2013; Hofius et al., 2011). \textit{acd11} is stressed from sphingolipid perturbations, an active immune response and the associated HD PCD, so if all of these stressors are cumulative and not being removed, it may accelerate cell death (Figure 3.1.1). A more in depth look at the pro-life, pro-death scenario by additive stressors in autophagy deficient mutants, is presented in the results section 3.3.

A large variety of agonists and endogenous stress signals results in the accumulation of ceramides in mammals, which often occur prior to morphological changes, suggesting that sphingolipids are involved in the cell death response (Hannun et al., 2000). Fluctuations in ceramide levels is sufficient to mimic many of the responses normally induced by other stressors (Hannun et al., 2000). Extraneous ceramide induces cell death in both plants and mammals, which can be prevented by the addition of sphingosine-1-phosphate, indicating that the balance between different sphingolipid species may determine cell fate (Liang et al., 2003; Cuvillier et al., 1996; Van Brocklyn et al., 2012). In this respect it is interesting that externally delivered C1P at low concentrations induces DNA synthesis in mammalian cells, and may thus act as a potential mitogen (Gomez-Munoz et al., 1997). Crystallization of ACD11, structural determination and transport assays show it to be a C1P and phyto-C1P transporter. But transporter function is dispensable for \textit{acd11 PCD}, as sphingolipid transport deficient versions complement the \textit{acd11} cell death phenotype, indicting that changes in ACD11-regulated sphingolipid
metabolism is not triggering the HR response (Simanshu et al., 2014; Petersen et al., 2008). If FB₁ induces PCD through sphingolipid modulation, it is different from the one induced in acd₁₁, as retromer doubles suppress acd₁₁ cell death but not FB₁ induced cell death (Figure 3.1.17). ACD₁₁ appears to be guarded by LAZ5 and sphingolipid metabolism is affected upon Pst infections, indicating that sphingolipids are either required for shaping the immune response or is manipulated by pathogens (Palma et al., 2010; Liang et al., 2003; Cuvillier et al., 1996; Gomez-Munoz et al., 1997; Peer et al., 2010; Pata et al., 2010). Overall levels of C₁P species are much higher in acd₁₁ than in NahG, indicating that sphingolipids are part of the immune response or following stress responses (Figure 3.1.15 and 3.1.16). Interestingly, as C₁P species also appear to accumulate in untreated acd₁₁ NahG compared to NahG, it shows that loss of ACD₁₁ transporter activity is responsible. Whether the accumulation is due to lack of transport and later metabolism of specific sphingolipid species remains to be seen, as it may also merely be a feedback mechanism further upstream of ACD₁₁-dependent pathways to compensate. If the lack of transport causes accumulation of C₁P in the Golgi or ER, it may induce organelle stress through modulation of membrane composition, which has also been observed for the human homolog of ACD₁₁, CPTP (Simanshu et al., 2013). Localization studies of ACD₁₁ are required to determine whether this idea should be further pursued.

Phyto-C₁P levels were affected by BTH treatment alone, making it harder to determine the exact role of ACD₁₁ transport on this particular sphingolipid species (Figure 3.1.16 and supplemental table 1). As levels in acd₁₁ were higher than in both treated an untreated NahG, showing that sphingolipid species accumulate over time, most likely in part of the continuing HR-associated PCD. But it is clear that loss of ACD₁₁-dependent transport induce accumulation of phyto-C₁P, as BTH treated acd₁₁ NahG samples were significantly higher than BTH treated NahG in most cases.

The changes in the sphingolipid profile following introduction of NahG in acd₁₁ show potential roles for C₁P and phyto-C₁P in shaping the defense response, while at the same time that acd₁₁-associated PCD is dependent on SA. ACD₅ may control levels of C₁P together with ACD₁₁, and while the perturbations are relatively minor, it may be necessary for full HR-associated PCD. Interestingly, knockdown of ceramide kinase in
human cancer cells also enhances apoptosis, and high extraneous C1P concentrations results in cell death, due to an increase in ceramide by the metabolizing effect on C1P by lipid phosphatases (Mitra et al., 2007). The loss-of-function inositolphosphorylceramide synthase mutant erh1 exhibit the same phenomenon as acd11 and acd5, as SA is required for triggering an immune response (Wang et al., 2008). Sphingolipid bases are up-regulated early during infections, and therefore it is possible that sphingolipids are merely defense signaling mediators and not required for cell death execution as such (Peer et al., 2010; Mackey et al., 2003). Therefore it may be that the sphingolipid responses are minor during an infection, but amplified through SA accumulation. That phyto-C1P levels in BTH treated acd11 NahG are higher than in BTH treated NahG supports this hypothesis (Supplemental table 1). A closer examination of the sphingolipid profile during HR-associated PCD in various SA-deficient backgrounds, may provide better determination of the validity of this idea, such as by using the acd11 eds5 and acd11 sid2 double mutants (Brodersen et al., 2005).

In conclusion, these results show a close genetic link between the retromer complex and immunity-associated cell death. By dissecting the contribution of the retromer complex in effector-triggered PCD, it is clear that there are unique features downstream of different types of R proteins and that cell death in LMMs can result from different molecular pathways.
3.2 The role of autophagy during HsFAPP2 knockdown

At a glance plants and mammals share quite a few similar features in the innate immunity, such as the presence of PRRs in the plasma membrane, NB-LRRs in the cytoplasm, and activation of MPK cascades, ROS accumulation and gene transcription downstream of pathogen detection (Nurnberger *et al.*, 2004). The human NB-LRR family is quite small with only little more than 20 members, compared to the 149 members in *Arabidopsis*, suggesting diversifying functions (NIBLRRS; Ye *et al.*, 2008; Franchi *et al.*, 2009). This may be partially explained by the way pathogens infect cells, as most plant pathogens are extracellular and many mammalian pathogens are intracellular and have to evade the adaptive immune system prior to infection. The most well described mammalian NB-LRR is NLRP3, which upon activation assembles the inflammasome. It is a multimeric structure that ultimately leads to activation of CASPASE-1 and the following proteolytic activation of the pro-inflammatory cytokine INTERLEUKIN-1 (IL-1), priming defense responses. NLRP3 is activated by a wide array of MAMPs and DAMPs, including bacterial and viral RNA, lipopolysaccharides and PGNs, which can explain why there are so few NB-LRRs in mammals (Franchi *et al.*, 2009; Jin *et al.*, 2010). Recently it was discovered that the NB-LRRs NOD1 and NOD2 recruits the autophagic machinery to the plasma membrane at the site of pathogen entry, limiting pathogen proliferation (Travassos *et al.*, 2010). It is well known that autophagy sequester and degrades invading pathogens, but direct activation depending on NB-LRR activation had not been shown previously (Travassos *et al.*, 2010; Nakagawa *et al.*, 2004; Ogawa *et al.*, 2005; Birmingham *et al.*, 2007).

Loss of ACD11 in *Arabidopsis* leads to NB-LRR dependent activation of HR-associated PCD, which most likely is dependent on autophagic cell death (Brodersen *et al.*, 2002; Palma *et al.*, 2010; Hofius *et al.*, 2009). A homolog of ACD11, het-c from *P. anserina*, is involved in incompatibility interactions that potentially leads to autophagy-dependent cell death (Pinan-Lucarre *et al.*, 2003; Saupe *et al.*, 1994). The c-terminal GLTP-domain of human FAPP2 also show homology to ACD11, although they seem to be involved in the transport of different sphingolipids, as ACD11 transport C1P and phyto-C1P, while FAPP2 mediates glucosylceramide transport from the TGN to the plasma membrane or between...
Golgi compartments (Simanshu et al., 2014; D’Angelo et al., 2007). Removal of ACD11 appears to affect sphingolipid metabolism, similar to FAPP2 when it is knocked down by siRNA (Figure 3.1.15; Simanshu et al., 2014; D’Angelo et al., 2007). Knockdown of FAPP2 also makes cells more sensitive to apoptotic inducers, indicating increased stress upon FAPP2-mediated transport (Tritz et al., 2009). Currently no observations have been made of NB-LRR-mediated detection of host protein loss in mammals, similar to what has been established as the guard hypothesis in plants (Jones et al., 2006).

To investigate the potential role of FAPP2 in regulation of PCD, Søren Skov and Morten Petersen knocked down FAPP2 using siRNAs and observed a significant loss of viability of Jurkat T cells, compared to scrambled siRNA controls (Manuscript in preparation). Large autophagosome-like vesicles after FAPP2 knockdown were observed by electron microscopy, and fluorescence microscopy also confirmed formation of ATG8/LC3 foci in an ATG6- and ATG7-dependent manner. Further, depletion of ATG6 or ATG7 along with FAPP2 significantly increased viability, indicating that FAPP2 regulates PCD in an autophagic-dependent manner. FAPP2 has previously been shown to be required for TGN to plasma membrane transport, and surprisingly Skov et al., found that siRNA targeting ATG7 also blocks transport in a similar fashion, indicating that FAPP2 may indirectly cause an autophagy-dependent block of membrane-targeted transport (Vieira et al., 2005; Yui et al., 2009; D’Angelo et al., 2007). The pro-life regulation by FAPP2 was also illustrated by the observation that FAPP2 mRNA accumulates in several cancer cell lines. These observations indicate that FAPP2 regulates cell death in an autophagy-dependent manner similar to ACD11.

### 3.2.1 Characterization of FAPP2 knockdown in HeLa cells

Activation of the autophagic machinery upon FAPP2 knockdown may either be a direct function as a pro-death executioner, or a general stress response (Kaushik et al., 2010; Lu et al., 2013). In order to differentiate between these two possibilities and to establish if FAPP2 knockdown also triggers autophagy-dependent cell death in other cell lines, HeLa cells were transfected with two siRNAs targeting FAPP2 (siFAPP2b, Skov et al.; and siFAPP2v,
LC3B is a human homolog of ATG8, and is often used as an indirect tool for observing autophagic up-regulation, as accumulation of autophagosomes depend on an ATG8 coat to target cargo for sequestration. As seen in figure 3.2.1, knockdown of FAPP2 in HeLa cells with either siFAPP2b or siFAPP2v did not appear to up-regulate both un-lipidated and lipidated forms of LC3B compared to a scrambled siRNA control (siControl), suggesting that either siRNAs have no effect on autophagy, or that FAPP2 is not knocked down properly. To verify that both siRNAs are indeed capable of targeting FAPP2 in HeLa cells, knockdown of two FLAG-tagged FAPP2 constructs were co-transfected along with the siRNAs. FLAG-FAPP2-WT encodes the WT FAPP2 cDNA sequence and should be targetable by both siRNAs, while FLAG-FAPP2-Mut contains a synonymous FAPP2 sequence mutation that hinders siFAPP2b binding. While FLAG-FAPP2-WT knockdown was observed by co-transfection with either of the siRNAs, the FLAG-FAPP2-Mut construct was not affected by siFAPP2b co-transfection (Figure 3.2.1). The scrambled siControl did not have an effect on FLAG-FAPP2 expression for either constructs, indicating that it is a proper control. Transfection with either of the FLAG constructs appeared to cause an increase in the balance between LC3B un-lipidated and lipidated forms, suggesting that FAPP2 has a cyto-protective role, or that expression of the exogenous construct is harmful to the cells.

**Figure 3.2.1:** Immunoblot against LC3B and FLAG epitopes in HeLa cells transfected with siRNAs against FAPP2 or cotransfected with FAPP2 siRNAs and a WT or mutated FLAG-FAPP2 construct. Scrambled siControl used as siRNA control. β-actin served as loading control. I and II indicates lipidated and un-lipidated forms of LC3B respectively.
While autophagy does not appear to be up-regulated upon siFAPP2 knockdown of HeLa cells, it is possible that it is stressful to them. Bafilomycin A1 is a lysosomal V-ATPase inhibitor that increases lysosomal pH and inhibits autophagy by blocking autophagosome-lysosome fusion (Yoshimori et al., 1991; Klionsky et al., 2012). Treatment of siFAPP2 transfected HeLa cells for 4 hours with bafilomycin A1 did not appear to cause a stronger accumulation of LC3B-II levels over siControl transfected cells, indicating that the level of stress is not increased upon FAPP2 knockdown. Increases in the LC3B-II form are not necessarily a direct indicator of an increased autophagic flux, but may merely be a blockage of autophagosome degradation (Rubinsztein et al., 2009; Klionsky et al., 2012). As the observations in Jurkat T cells may be attributed to this scenario and that FAPP2 appears to be involved in sphingolipid metabolism and thus endomembrane composition, the degradative flux was examined after FAPP2 knockdown. Treatment induced turnover of isotope tagged long-lived proteins can be used as a good indicator for up-regulation of the autophagic machinery, as one can determine its contribution to overall protein flux, by specifically blocking the pathway. As shown for figure 3.2.3, the difference between control and bafilomycin A1 treated samples were similar for siControl and siFAPP2b siRNAs, indicating that the autophagic flux was similar for both treatments. Surprisingly, the overall degradative change for both control and Bafilomycin A1 treated samples were much higher in the siFAPP2b treated samples. This suggests that other degradative pathways, most likely proteasomal, are active upon FAPP2 knockdown.

Figure 3.2.1: Immunoblot against the LC3B epitope in duplicate HeLa cells transfected with siRNAs against FAPP2, with and without 4 hours of Bafilomycin A1 treatment. Scrambled siControl used as siRNA control. β-actin served as loading control. I and II indicates lipidated and un-lipidated forms of LC3B respectively.
Since ACD11 is guarded by LAZ5 and therefore involved in defense against pathogens, it is hypothetically possible that FAPP2 is also required during the immune response in human cells. To test this hypothesis, total RNA was extracted from FAPP2 knocked down cells and expression of markers specific for an activated immune response was analyzed. As a positive control, ie-DAP, an agonist specific for the NB-LRR NOD1 was used at three different concentrations. As shown in figure 3.2.4, no immediate increase in expression of the stress-induced TF CHOP or the HSP Grp78 was observed even for 100 μg/mL concentrations of the NOD1 agonist, questioning the validity of these two markers for NB-LRR activation. High concentrations of the NOD1 agonist activated the expression of IL-6 and IL-8, but no change in expression was seen for siFAPP2b and siFAPP2v compared to the control siRNA. This data further indicates that the FAPP2 targeting siRNAs does not induce a stress response.

![Figure 3.2.3: Long lived protein degradation in control and Bafilomycin A1 treated samples, after siRNA knockdown by siFAPP2b compared to a scrambled control. Values are plus/minus standard deviation calculated from 2 replicates.](image)
3.2.2 Discussion of FAPP2 in autophagic regulation

The GLTP domain of FAPP2 show homology to the *Arabidopsis* C1P transporter ACD11. Skov et al., (Manuscript in preparation) observed activation of autophagic up-regulation upon FAPP2 siRNA knockdown in Jurkat T cells, indicating a role for glucosylceramide transport in cell death responses. The data presented here suggests that removal of FAPP2 in HeLa cells does not have a measurable effect on autophagy (Figure 3.2.1, 3.2.2 and 3.2.3). siRNA mediated FAPP2 knockdown was confirmed by depletion of a FLAG-FAPP2 construct, indicating that transfection protocols were functioning correctly (Figure 3.2.1). From figure 3.2.1 it does appear that over-expression of FAPP2 assert a protective role or induces stress, but wether this is directly related to the protein or merely a side effect of the transfection protocol needs to be confirmed. If the up-regulation of the LC3B-II isoform is indicative of a cyto-protective role, it would be a confirmation of the observations of Skov et al., that levels of FAPP2 mRNA are higher in several cancer cell lines. These data does not confirm that FAPP2 is fully knocked down, so it is possible that minute levels of FAPP2 mRNA transcript are sufficient in maintaining FAPP2 functionality. As a FAPP2

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specific antibody was not available to me at the time, protein levels of endogenous FAPP2 could not be analyzed. Since a functional polyclonal antibody is available, it may be possible to confirm protein removal (Godi et al., 2004).

Transport of specific components from the TGN to the plasma membrane is inhibited upon FAPP2 knockdown and Skov et al. found that knockdown of ATG6 or ATG7 also blocked transport in a similar fashion, implying that autophagy is required for FAPP2-mediated transport (Vieira et al., 2005; Yui et al., 2009; D’Angelo et al., 2007). FAPP2 contains an N-terminal PH domain, which enables it to bind to PtdIns(4)P containing membranes (Godi et al., 2004). Whether this domain also allows it to bind to PtdIns(3)P in autophagosomal membranes is currently unknown. Another possibility is that blockage of autophagy results in depletion of endosomal membrane, due to accumulation of autophagosomes that are not being turned over. This would potentially block vesicle-mediated transport from the TGN, thus mimicking FAPP2 depletion. In MDCK cells, FAPP2-mediated transport is only inhibited during apical transport, and not basolateral, which would make it possible to distinguish between a general endosomal response or a FAPP2-specific response, upon FAPP2 and ATG knockdown (Vieira et al., 2005).

Cell death pathways appear to be differently regulated in various cell types, which may be the reason for the PCD response observed upon FAPP2 knockdown in Jurkat T cells, but not in HeLa cells (Nikoletopoulou et al., 2013). FAPP2 depletion did not appear to affect levels of either of the two stress response markers CHOP and Grp78, nor IL-6 and IL-8, indicating no role for FAPP2 in immunity-associated cell death regulation (Figure 3.2.4). The catabolic autophagic flux appeared to be unchanged upon siFAPP2 treatment, further confirming that FAPP2 depletion does not increase autophagic cell death in HeLa cells (Figure 3.2.3). For some currently unknown reason, it appeared that overall protein turnover was increased after FAPP2 knockdown. Repetition of the assay and the addition of the second FAPP2 targeted siRNA will help confirm this result.

Overall these results indicate that FAPP2 does not regulate immunity-associated cell death in HeLa cells, although results are too preliminary to rule out a definite contribution.
3.3 Autophagy during immunity-associated cell death

Autophagy is a catabolic process required for degradation and recycling of nutrients, and is widely considered a general response to various forms of stress and vital for maintaining cellular homeostasis (Ryter: 2013vv, Kaushik et al., 2010; Lu et al., 2013; Robaglia et al., 2012). In accordance with this, Arabidopsis autophagy loss-of-function mutants show early senescence under nutrient limiting conditions and increased sensitivity to external stimuli such as heat stress (Supplemental figure 7.2; Onodera et al., 2005; Zhou et al., 2013). Apart from acting as a pro-life pathway, autophagy can also be induced as a cell death pathway used for shaping vascular structures (Kwon et al., 2011; Kwon et al., 2010). In addition the pathway is well documented for being required for HR-triggered PCD, where most sources have observed autophagy as a pro-life pathway required to contain lesions from spreading from the original entry point, several days after the initial infection in older atg mutants (7-8 weeks old; Yoshimoto et al., 2009; Liu et al., 2005; Patel et al., 2008). Nonetheless, autophagy has also been shown to be required as a pro-death defense against biotrophic pathogens, as Pst DC3000 carrying avrRpm1 trigger autophagic cell death (Hofius et al., 2009; Kwon et al., 2013). These apparent opposite observations are further challenged by observations by our group (Hofius et al., 2009) and Yoshimoto et al. (2009), that younger atg mutants (4-5 weeks old) appear to be able to contain the spreading lesion, after Pst DC3000 avrRpm1 infection, indicating that age has a profound effect on HR-development.

Yoshimoto et al. (2009) established that SA levels and the associated defense- and senescence markers PR1, PR2 and SAG12 were up-regulated in both atg2 and atg5 mutants, and the expression of the marker genes correlated with the onset of early senescence compared to WT plants. More specifically, genetic markers and early senescence were induced earlier in atg2 mutants, than in atg5 mutants. Both the early senescence phenotype and SA-dependent gene expression could be reversed by expression of NahG or the introduction of sid2 or npr1 knockouts into the atg mutants (Supplemental figure 7.3). More importantly, Yoshimoto et al. (2009) found that suppression of the SA-dependent features were also observed by the introduction of these deficient backgrounds.
into the \textit{atg} mutants. Exogenous application of BTH restored the spreading lesion phenotype in \textit{atg5 NahG} and \textit{atg5 sid2} double mutants, but not in \textit{atg5 npr1}. These observations led Yoshimoto \textit{et al.}, (2009) to conclude that autophagy control plant senescence and immunity-associated cell death, by regulating a negative feedback loop through NPR1 to modulate SA signaling.

3.3.1 NPR1 is not a positive regulator of RPM1-triggered cell death

The apparent contradictory observations that autophagy is needed both as a pro-life pathway to contain spreading lesions, and also as a pro-death pathway, can potentially be explained by when and where the observations are made. Spreading lesions are first observed several days after infection and outside of the initial infection site, while pro-death autophagy-dependent HR-development is observed in the immediate hours following infection. Since NPR1 has previously been implicated in restricting \textit{avrRpm1}-triggered cell death, its role in HR-triggered cell death was investigated (Rate \textit{et al.}, 2001; Fu \textit{et al.}, 2012). As seen in figure 3.3.1, Pst DC3000 \textit{avrRpm1} induced cell death as monitored by ion leakage, was significantly higher in the \textit{npr1} knockout mutant than in WT, which is in accordance with previous observations (Rate \textit{et al.}, 2001). This observation show that NPR1 is not required for promoting \textit{avrRpm1}-induced cell death. \textit{atg5} on the other hand, displayed a subtle but significant suppression of cell death, as has also been observed before, suggesting the pro-death nature of the autophagic machinery in HR PCD (Hofius \textit{et al.}, 2009). Interestingly, introduction of the NPR1 loss-of-function allele into \textit{atg5} also displayed cell death at the level of \textit{npr1}. This again confirms that NPR1 is not required for triggering HR cell death. This is also in accordance with a weaker HR seen in plants that over-express NPR1 (Rate \textit{et al.}, 2001). In order to verify that these observations were not limited to \textit{atg5} alone, but a general feature of \textit{atg} mutants, the assay was repeated in the stronger autophagy-deficient mutant \textit{atg2} (Yoshimoto \textit{et al.}, 2009; Wang \textit{et al.}, 2011). ATG5- and ATG7-independent autophagy has been reported before, which may account for the stronger autophagy-deficiency observed for \textit{atg2} (Nishida \textit{et al.}, 2009). As expected, \textit{atg2} was observed to suppress RPM1-triggered cell death even more strongly than \textit{atg5},
compared to WT (Figure 3.3.2). As observed for atg5 npr1, introduction of npr1 into atg2 restored cell death, although not to the extent of npr1. It is possible that loss of autophagy induces other PCD pathways to compensate, and that NPR1 is required for keeping these pathways in check. Cross talk is well documented in mammals, and loss of autophagy can induce apoptosis under certain conditions (Nikoletopoulou et al., 2013). Similar observations were made for suppression of avrRpm1-triggered cell death in older atg mutants, and extending the ion leakage analysis beyond 7 hours did not yield any significant changes (Data not shown). Also, to ensure that the differences in cell death suppression were not due to different growth rates of Pst DC3000 avrRpm1 in different genotypes, bacterial growth assays were carried out. Bacterial numbers revealed no significant differences 6 hours after infection, except in the rpm1 knockout mutant, proving that there was no direct correlation (Supplemental figure 7.3).

**Figure 3.3.1**: Ion leakage assay of 6 weeks old Col-0 WT, rpm1, atg5, npr1 and atg5 npr1 plants, after inoculation with Pst DC3000 avrRpm1. Mean and standard deviations were calculated from four or six discs per treatment with 3 or 4 replicates within an experiment. Pairwise comparisons at the last time point post infection for means versus WT, were performed using one-way ANOVA test followed by Holm-Šidák post-hoc test. *, P ≤ 0.05, **, P ≤ 0.01; ***, P ≤ 0.001.
To support the ion leakage observations, leaves were stained by trypan blue after being infected with \textit{Pst} DC3000 \textit{avrRpm1}, and analyzed by light microscopy using a lower dose of \textit{Pst} DC3000 \textit{avrRpm1} so not to completely overwhelm the tissue. Trypan blue is taken up by dead or dying cells, as it cannot cross an intact plasma membrane. Compared to WT, \textit{atg2} was observed to exhibit fewer dead cells, similar to the ion leakage results (Figure 3.3.3). While \textit{atg2 npr1} leaves had a number of dead cell similar to WT, \textit{npr1} had was observed to have even more dead cells, which is in agreement with Rate and Greenberg (2001). These observations reveal that there are genetic and temporal differences between the cell death observed during immediate HR-development, and the spreading lesions in the days following infection in older \textit{atg} mutants.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure3.3.2.png}
\caption{Ion leakage assay of 6 weeks old Col-0 WT, \textit{rpm1}, \textit{atg2}, \textit{npr1} and \textit{atg2 npr1} plants, after inoculation with \textit{Pst} DC3000 \textit{avrRpm1}. Mean and standard deviations were calculated from four or six discs per treatment with 3 or 4 replicates within an experiment. Pairwise comparisons at the last time point post infection for means versus WT, were performed using one-way ANOVA test followed by Holm-Šidák post-hoc test. *, P ≤ 0.05, **, P ≤ 0.01; ***, P ≤ 0.001.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure3.3.3.png}
\caption{Photos of leaves from Col-0 WT, \textit{npr1}, \textit{atg2}, \textit{atg2 npr1} and \textit{rpm1} plants stained with lactophenol-trypan blue, 12 hours after \textit{Pst} DC3000 \textit{avrRpm1} infection. Size bar represents 2,5mm.}
\end{figure}
3.3.2 Autophagic markers is suppressed by NPR1 loss-of-function

These results raise further questions; Why is early senescence in atg mutants suppressed by mutations in NPR1 and SID2 (Supplemental figure 7.3), and why are atg mutants hyper-sensitive to BTH and avirulent infections? A possible answer could be due to the very nature of autophagy. As a catabolic pathway required for basal degradation of protein aggregates and dysfunctional organelles, atg mutants are prone to accumulate cellular ‘debris’ as they get older. Secondary metabolites, ROS and lipid peroxides will start to concentrate due to the lack of autophagy, and these induce stress responses such as SA and PR proteins (Anderson et al., 1998; Xiong et al., 2007; Chaouch et al., 2010). This stress would reinforce itself in autophagy-deficient mutants, resulting in earlier senescence. As SA induced defense responses require NPR1 for PR production, such a positive feedback loop would be suppressed by loss-of-function mutations in NPR1 (Supplemental figure 7.2).

To assay whether it is possible to detect NPR1-dependent differences in the atg2 mutant background, immunoblotting for ATG8a was carried out. Similar to NBR1, ATG8 accumulate in backgrounds with blocked autophagy, as it associates with the autophagosomal membrane, but does not become degraded and thus turned over. In 3 weeks old mutants of the npr1 background, minor build up of ATG8a was detected, due to normal autophagic flux (Figure 3.3.4A). In contrast, young atg2 mutants accumulated large amounts of ATG8a, which were similar to that of atg2 npr1 mutants. Surprisingly, slightly more ATG8a was detected in atg2 at 6 weeks and 9 weeks, contrary to atg2 npr1 which remained relatively constant (Figure 3.3.4A). NBR1 contains a LIR domain that allows it to bind to ATG8, and thus act as a cargo adapter associated with the autophagosome, before being degraded in the vacuole (Svenning et al., 2011; Zhou et al., 2013). Strong accumulation of NBR1 was observed even in young atg2 and atg2 npr1 mutants, compared to npr1, indicating block of the autophagic flux (Figure 3.3.4B). A slight increase was again observed for NBR1 in 6 weeks old atg2, but this was much more pronounced in 9 weeks old mutants. In comparison atg2 npr1 mutants were observed to remain relatively comparable at all time points. These data strongly suggest that the introduction of npr1
into \textit{atg2} lowers the autophagic flux, as seen by higher accumulation of ATG8a and NBR1 over time compared to \textit{atg2}. It should be noted that no difference between WT and \textit{npr1} was observed (Figure 3.3.6, untreated samples).

**Figure 3.3.4:** Immunoblot detection of (A) ATG8a and (B) NBR1 accumulation from \textit{npr1}, \textit{atg2 npr1} and \textit{atg2} plants, 3, 6 and 9 weeks old. Coomassie staining of the large subunit of RuBisCO serve as loading control.

### 3.3.3 Ubiquitinated substrates accumulate over time in \textit{atg} mutants

Apart from the LIR domain, \textit{AtNBR1} also has two UBA domains where one of them can bind ubiquitin, meaning that NBR1 can interact with ubiquitinated proteins destined for degradation. Since bulk degradation of proteins is blocked in \textit{atg} mutants, ubiquitinated protein aggregates should accumulate in these backgrounds. To see if there was a correlation between ubiquitinated substrates and autophagy deficiency, 3 weeks old WT, \textit{npr1}, \textit{atg2}, and \textit{atg2 npr1} plants were assayed. As shown in figure 3.3.5, no major differences were observed between genotypes for young plants (Supplemental figure 7.4A). However, at 6 weeks a major accumulation of ubiquitinated proteins were observed in \textit{atg2} knockout plants compared to the other genotypes, especially \textit{atg2 npr1} (Supplemental figure 7.4B). At 9 weeks this difference was even more pronounced (Figure 3.3.5). This indicates that a slow but steady build up of proteins is occurring in autophagy-deficient backgrounds, and that lack of a functional SA pathway dampens this from happening. To ensure that this was not just an artifact from the anti-ubiquitin antibody
used for the immunoblots, a secondary antibody was assayed and produced similar results (Supplementary figure 6.4).

During an immune response to *Pst* DC3000 avrRpm1 plants induce SAR, a heightened state of defense in distal tissues, which leads to local increases in SA and defense gene transcription in uninfected tissues (Maleck et al., 2000). Both PR gene expression and genes required for induction of the secretory machinery during SAR, are controlled by NPR1. The increased secretion of peptides requires ER-specific proteins that help ensure proper folding of nascent chains such as chaperones (Wang et al., 2005; Cao et al., 1994). As the load increases on the ER, autophagy is induced to selectively degrade parts of the ER, to remove misfolded proteins (Liu et al., 2012). Since SAR can be artificially induced by application of BTH, it is possible to mimic an infection. This should in turn lead to accumulation of unfolded proteins that are ubiquitinated, and depending on autophagy to be degraded. To assay this possibility, 6 weeks old plants were treated with BTH and immunoblotted for NBR1 and ubiquitin accumulation. As seen in figure 3.3.6A, induction of SAR enhances the accumulation of NBR1 in *atg2* mutants compared to *atg2 npr1*. Since NPR1 is required to mediate BTH signaling, no change was observed in the double mutant. This is also apparent if WT is compared to *npr1*, as a slight increase is seen. Since the WT has normal autophagic degradation, NBR1 accumulation does not occur to

![Figure 3.3.5](image_url): Immunoblot detection of ubiquitin in 3, 6 and 9 weeks old WT, *npr1*, *atg2 npr1* and *atg2* plants. Amido black staining of the large subunit of RuBisCO serve as loading control. Anti-ubiquitin antibody from Dako.
the same extent as in \textit{atg2}. Similarly to what is observed for NBR1, ubiquitinated substrates accumulate much more in the BTH treated \textit{atg2} mutant (Figure 3.3.6B).

![Figure 3.3.6: Immunoblot detection of (A) NBR1 and (B) ubiquitin in 7 weeks old WT, \textit{npr1}, \textit{atg2} \textit{npr1} and \textit{atg2} plant, after 24 hours of treatment with mock or 100 μM BTH. Amido black staining of the large subunit of RuBisCO serve as loading control. Anti-ubiquitin antibody from Dako.](image)

### 3.3.4 \textit{npr1} lowers the stress load in \textit{atg} mutants

An avirulent immune response triggers a strong up-regulation of PR proteins that needs to be folded correctly, and it is evident that lack of a proper ER stress response can be detrimental. SA treatment of \textit{bip2}, an ER residing chaperone, induces tissue collapse and cell death due to excessive ER stress, which can be rescued by a loss-of-function allele of NPR1 (Yorimitsu \textit{et al.}, 2006; Wang \textit{et al.}, 2005). Intriguingly, autophagy-deficient mutants are hyper-sensitive to ER stress (Bernales \textit{et al.}, 2006; Yorimitsu \textit{et al.}, 2006). These observations explain why \textit{npr1} can suppress both spreading lesions following an immune response as well as senescence-associated tissue collapse, since both situations trigger ER stress (Wang \textit{et al.}, 2005). The UPR is activated upon ER stress, to ensure expression of chaperones and similar proteins required for dealing with nascent peptide chains. One of the UPR pathways rely on two components normally embedded in the ER membrane,
namely the ribonuclease IRE1 and the transcription factor bZIP60 that regulates ER stress specific genes. When stress increases in the ER, IRE1 splices the bZIP60 mRNA transcript, so that a new form without the transmembrane domain is allowed to move to the nucleus. This spliced form of the bZIP60 transcript is therefore a good indicator for the state of stress in the ER (Iwata et al., 2008; Iwata et al., 2005; Nagashima et al., 2011; Deng et al., 2011; Mishiba et al., 2013; Moreno et al., 2012). So RT-PCR of the spliced and unspliced bZIP60 transcripts were analyzed to establish the relative level of ER stress in the atg2 and atg2 npr1 backgrounds. As figure 3.3.7 shows, no major differences were observed for the unspliced bZIP60 transcript between all the genotypes when looking at plants that were 6 weeks old. However, spliced bZIP60 transcript was clearly expressed to higher levels in atg2 than WT, and even higher than in both npr1 and atg2 npr1. Looking at plants that was one week older (7 weeks), this difference was even more pronounced, and clearly this indicates that the level of ER stress is much lower in npr1 backgrounds. This is additional evidence for how loss-of-function of NPR1 can rescue spreading cell death in uninfected, but SAR triggered, tissue, as defense gene expression is not activated, and ER stress is much lower (Wang et al., 2005).

Figure 3.3.7: RT-PCR of unspliced and spliced bZIP60 mRNA levels in Col-0 WT, npr1, atg2 and atg2 npr1, in 5 and 7 weeks old plants. Actin serves as loading control.
3.3.5 Additive stressors kill autophagy-deficient mutants

TM is a strong inducer of ER-stress, since it inhibits protein N-glycosylation, and blocks exit for a large number of nascent peptide chains originally destined for secretion. Since autophagy is required for degradation of the ER during ER stress, TM treatment triggers autophagy (Liu et al., 2012). Since these data indicate that cellular homeostasis is compromised in atg mutants, and that autophagy-deficient mutants are hyper-susceptible to external stressors, it should be possible to combine stressors and produce lethal effects in young atg mutants. To examine this hypothesis, WT, atg2, atg2 npr1 and npr1 seeds were germinated on MS plates containing TM, BTH or both at the same time, to induce an artificial defense response on top of ER stress. As seen in figure 3.3.8, young atg2 plants were unable to cope with the additive stress, contrary to the atg2 npr1 double mutant which was relatively unaffected as they don’t respond to the BTH or SA-dependent feedback from the UPR induced by TM. Similar observations were made for atg5 and atg5 npr1 (Supplemental figure 7.5). As observed in figure 3.3.8, atg2 seeds germinated less successfully on BTH and the combined TM and BTH treatments, than on mock treatment. To rule out that the few atg2 seedlings were not exposed to higher concentrations than the other genotypes, seedlings were allowed to germinate on MS without any additives, and then transferred to MS plates with BTH and TM after germination. Handling the genotypes this way did not change the outcome, as all genotypes other than atg2 handled the stress in the same way (Supplemental figure 7.6). This strongly indicates that stress in autophagy-deficient mutants can be additive and lethal.
Finally, in an attempt to mimic the uncontrolled spread of cell death seen in older atg mutants after avirulent infection, in young atg mutants, the additive stress approach was used. Leaves of all four genotypes were injected with TM in one side, allowed to cope with ER stress over 5 days, and then infiltrated in the opposite side of the leaf with Pst DC3000 avrRpm1 (Figure 3.3.9). After 4 days, a clear but contained lesion had developed where RPM1-triggered cell death had occurred, in WT, npr1 and atg2 npr1. This was in stark contrast to atg2, where entire leaves had succumbed to spreading cell death in the same time frame. Individual treatment with Pst DC3000 avrRpm1 was contained in the atg2 mutant, similar to the other genotypes, while TM had no visible effect (Figure 3.3.9). Clearly stress in the atg2 mutant was still present, even several days later, and an infection on top pushed the tissue past conditions required for survival. Therefore, when additive stress is happening in autophagy-deficient mutants, here exemplified by ER stress and an immune response, the outcome will be cell death.

Figure 3.3.8: Photos of 4 weeks old Col-0 WT, npr1, atg2 and atg2 npr1 plants grown on MS plates supplemented with 0.00005% DMSO (Mock), 5 ng/mL tunicamycin, 50 µM BTH or 5 ng/mL tunicamycin and 50 µM BTH.
3.3.6 Discussion of autophagy during immunity-associated stress

Autophagy is catabolic pathway and recycling process required for the removal of accumulating cellular debris that would otherwise be detrimental to cell survival. In addition, the pathway is well known to combat deleterious stress, such as the presence of dysfunctional organelles, heat- or ER stress (Liu et al., 2012; MacVicar et al., 2013; Zhou et al., 2013). Here I show that additive stress causes additional harm in autophagy deficient backgrounds, and that loss-of-function mutations in the central SA response mediator NPR1 can suppress otherwise lethal stress (Figure 3.3.8 and 3.3.9). The data presented here provides evidence that the cell death inside and outside of infected areas in older autophagy-deficient mutants, is genetically different and that NPR1-dependent pathways are indirectly responsible for cell death outside the infected areas. A possible explanation is that the initial infection triggers a SAR response in non-infected areas, and when cellular homeostasis is compromised, as observed for older autophagy deficient mutants, then tissue collapse will occur (Figure 3.3.4, 3.3.5 and 3.3.9).

Less cell death is observed in atg2 mutants after infection with *Pst* DC3000 avrRpm1, and surprisingly introduction of *npr1* partially restore this phenotype (Figure 3.3.1, 3.3.2 and 3.3.3). Since atg2 npr1 double mutants are also autophagy deficient, NPR1
must suppress autophagy-independent cell death pathways. While no proof is presented on this hypothesis, evidently HR PCD may be executed by more than one pathway in plants (Mackey et al., 2002; Hofius et al., 2009; Coll et al., 2010; Hatsugai et al., 2009; McLellan et al., 2009; Gilroy et al., 2007). However, these observations suggests that autophagy does not regulate a negative feedback loop through NPR1 (Yoshimoto et al., 2009). If this theory is correct, then NPR1 should regulate autophagy in one manner inside infected areas, a second manner outside of infected areas, and at the same time differently for old and young plants.

Autophagy-deficient plants appear to suffer from early senescence in part from an accumulation of ubiquitinated proteins and increasing ER stress, leading to SA build up (Figure 3.3.5; Yoshimoto et al., 2009). NPR1 is such a central regulator of stress through SA signaling that introducing a loss-of-function mutation is sufficient in rescuing a wide array of stress that are reinforced through feedback loops. It is important here to emphasize that ubiquitin and ER stress are only two examples of stressors, and that other potential ‘debris’ may accumulate, such as lipids or organelles, although they may not cause harm to the same extent (Figure 3.3.8 and 3.3.9). Therefore, it is apparent that proper controls have to be used to analyze the effects on autophagy-deficient mutants. It also means that even though autophagy is up-regulated in seemingly unrelated backgrounds, it does not necessarily indicate an increased pro-death degradative flux. An autophagic flux to maintain homeostasis is present even in the absence of stress, so distinguishing between a proper autophagic response and background steady-state levels are crucial.

Taking this information into account, it is important that future research is carried out in as young autophagy-deficient plants as possible, as not to conclude on effects that are secondary and not directly related to the autophagic machinery per se. One possible way of addressing this matter is to establish new tools, such as inducible atg knockout mutants that can be triggered shortly before experiments, so that lifelong accumulation of stress is not masking results. Stress responses in plants are complex and often involve a lot of cross talk, so it is dependent on the setup of individual experiments on which controls are most suitable to use. The current set of tools for working on autophagy is still severely limited in plants compared to mammals and yeast, so it will be difficult to draw certain
conclusions concerning to autophagy in the forceable future, until the regulatory machinery is more well established.
4. Concluding remarks

The data presented here in this thesis show various aspects of innate immunity and associated cell death, along with a deeper insight into the workings of the autophagic pathway. The regulatory machinery of acd11-associated PCD is quite complex, making it difficult to determine how different pathways contribute, and it is clear from analyses with vps35 double knockout mutants that different R proteins, including LAZ5, regulate cell death through different regulatory nodes. Further confirmation of the genetics behind retromer-dependent regulation of plant endosomal trafficking will help to establish how it is involved in cell death, either directly or indirectly.

Looking at cell death suppression in retromer knockouts, it seems that autophagy assert a strong pro-death role during effector-triggered PCD, and somehow due to retromer mis-regulation, become non-functional in retromer knockout backgrounds. Autophagy can play the role of a protector but also that of a persecutor, as is apparent in the vps35a vps35c acd11 background, that die even faster than acd11. This difference makes autophagy an even more interesting pathway to work on, as conclusions have to be very carefully examined and backed up by convincing data. The current set of tools in plants for examining the genetics controlling the autophagic machinery is lacking, so the future poses great challenges to scientists working on deciphering PCD pathways. But at least I hope they will have as much fun as I had working with it.

-David Munch
5. Materials & methods

Apart from the long-lived protein degradation assay and qRT-PCR following siFAPP2 treatments (Figure 3.2.3 and 3.2.4), all assays were repeated at least twice.

5.1 Seeds material, sterilization technique and growth conditions

**Soil growth:** Seeds were incubated for 5 minutes in 70% EtOH, followed by two washes in sterile H₂O and resuspension in 0.1% agar, before being vernalized for 2-3 days at 4°C. Seeds were put on soil (K-jord, Weibull, Sweden) supplemented with 20% vermiculite (Skamol), 20% Perlite (Nordisk Perlit Aps), and 0.3% fertilizer (Osmocote Plus, Scotts Europe B.V) in Percival Scientific growth chambers (150 mE/m²/s and 70% relative humidity). Plants were grown under short day conditions (8 hours light, 16 hours darkness) at 21°C unless otherwise specified.

**Plate growth:** Seeds were incubated for 5 minutes in 1.3% sodium hypochlorite supplied with 0.02% Tween20, followed by 5 minutes incubation in 70% EtOH. Seeds were then washed twice in sterile H₂O, resuspended in 0.1% agar and distributed on sterile plates before being vernalized for 2-3 days at 4°C. Plates were then transferred to 21°C under long day light conditions (16 hours of light; 8 hours of darkness).

MS-media: 4.4 g/L Murashige & Skoog salt mixture, 10 g/L sucrose, 7.3 g/L agar, with pH adjusted to 5.7 with 1M KOH.

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5.2 DNA extraction

Modified from (Edwards et al., 1991). Approximately 0.5 cm² plant tissue was put in an eppendorf tube with 250 µL Edwards extraction buffer, and ground using a mechanical pestle. After spinning 15 minutes at 22,000g, 200 µL supernatant was transferred to a new eppendorf tube containing 200 µL isopropanol. Samples were then spun 10 minutes at 22,000g. Supernatant was removed, allowed to dry 15 minutes in a flow hood and pellet was then resuspended in 40 µL TE-buffer.

Edwards extraction buffer: 200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS.

5.3 Genotyping by polymerase chain reaction

**Homemade PFU, master mix**: 0.5 µL 10 mM dNTP mix, 2.5 µL 10x PFU-buffer, 0.25 µL of each primer, 1.25 µL DMSO, 1 µL 25 mM MgCl₂, 1 µL PFU polymerase, 0.75 µL DNA template, 17.50 µL H₂O.

**Standard PCR program**: Denaturing for 30 seconds at 96°C, annealing for 30 seconds at 58°C, and extension for 90 seconds at 72°C. 35 cycles.

PCR products were run on 1% agarose in 0.5x TBE gels, supplied with 0.05 µg/mL ethidium bromide. Gels were run at 125-180V and UV-light was used for visualization of DNA. DNA EcoRI/HindIII (Fermentas), 1000bp (Fermentas) or 100bp (Fermentas) DNA gene markers were used for determining approximate size.

**lsl1-2 WT PCR program**: Thermo Scientific, Phusion High-Fidelity DNA Polymerase: Denaturing for 30 seconds at 96°C, annealing for 30 seconds at 58°C, and extension for 90 seconds at 72°C. 35 cycles.

**lsl1-2 mutation PCR program**: Homemade PFU master mix: Denaturing for 20 seconds at 94°C, annealing for 30 seconds at 58°C, and extension for 60 seconds at 72°C. 35 cycles.
**Thermo Scientific, Phusion High-Fidelity DNA Polymerase master mix:** 0,5 μL 10 mM dNTP mix, 5 μL HF-buffer, 0,25 μL of each primer, 0,25 μL Phusion High-Fidelity DNA Polymerase, 0,75 μL DNA template, 18 μL H2O.

### 5.4 Real-time polymerase chain reaction

Total RNA was extracted using Omegas Plant RNA kit and cDNA synthesized using Life SuperScript III Reverse Transcriptase kit. Unspliced and spliced bZIP60 transcript levels were analyzed by RT-PCR as previously described (Nagashima et al., 2011; Deng et al., 2011)

### 5.5 Genotyping primers

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 | TTC ATA AGC AAC AGG CTC AAG ATC ACA | X | X  
 | TGG TTC ACG TAG TGG GCC ATC G | | X  

5.6 Bacteria and growth conditions

Bacteria were kept at -80°C in 15% glycerol, and were for assays grown overnight in 4 mL liquid NYG medium supplied with 25 ng/mL kanamycin and 100 μg/mL rifampicilin dissolved in methanol, at 28°C at 200 RPM. The overnight culture was added to a 50 mL liquid NYG supplied with 25 ng/mL kanamycin and 100 μg/mL rifampicilin dissolved in methanol, and shaken at 28°C, 200 RPM, for 2 hours. Bacteria were spun 7 minutes at 4000g, washed in 10 mM MgCl₂, spun again, and resuspended in 5 mM MgCl₂. Bacteria were then suspended to specific concentrations suitable for each assay, where the optical density (OD) at 0,1 corresponds to approximately 10⁸ CFU/mL.

For infection assays, virulent Pst DC3000 were used, or avirulent carrying avrRpm1, avrRpt2 or avrRps4.

NYG-media: 5 g/L bactopeptone, 3 g/L yeast extract, 20 g/L glycerol, 10 g/L agar.
5.7 Bacterial growth assay

Modified from Mackey et al., 2003. For bacterial growth determination *Pst* DC3000 concentrated to OD600 of 0.0001 was used (10^5 CFU/mL). Whole leafs were infiltrated with a syringe, and for time point 0 and 3 days, 3-6 leaf discs, each 0.4 cm in diameter, were ground in 1 mL 10 mM MgCl₂ with a mechanical pestle. 3-4 replicates were made for each genotype. For each replicate a 10-fold dilution series from 1:10^0 to 1:10^5 were made, and 20 μL of each were plated on a NYG plate supplied with 25 ng/mL kanamycin and 100 μg/mL rifampicilin dissolved in methanol, and grown for 2 days at 28°C, followed by the counting of CFUs and statistical analyses.

5.8 Cell death quantification by ion leakage upon bacterial infection

Modified from Mackey et al., 2003. For cell death quantification by ion leakage, *Pst* DC3000 concentrated to OD600 of 0.1 was used (10^8 CFU/mL). Whole leafs were infiltrated with a syringe, and 4-6 leaf discs, each 0.6 cm in diameter, were put in 4 mL Mili-Q water. 3-4 replicates were made for each genotype. For each time point the conductivity (μSiemens/cm) of the water was measured.

5.9 Leaf lesion development following FB1 injection

Modified from Stone et al., 2000 and Kuroyanagi et al., 2005. 4 weeks old plants grown under long light conditions (16 hours light, 8 hours darkness) were infiltrated in lower leafs with 0.5% methanol (Mock) or 10 μM *FB₁* dissolved in 0.5% methanol. Plants where then grown 7 days and photos were taken.

5.10 Leaf lesion development following TM and *Pst* DC3000 injections

One side of leaves from 4 week old plants were infiltrated with 100 ng/mL TM, followed by an injection in the opposite side of the leaf 5 days later, with avirulent *Pst* DC3000
avrRpm1 at 2 x 10^7 CFU mL^-1. Representative photos were taken 4 days after the last injection.

5.11 Additive stressors in plate growth

Seeds germinated on MS plates were supplemented with 0.00005% (v/v) DMSO (Mock), 0.005 µg/mL tunicamycin, 50 µM BTH or 5 ng/mL tunicamycin and 50 µM BTH.

5.12 Cell death induction by BTH

Plants were sprayed with 100 µM BTH three times with 3 days in between, and left out of the incubator until all liquid on the leaves had evaporated, and then moved back to incubator.

5.13 Plant immunoblotting

Total proteins were extracted in urea buffer (4M urea, 100mM DTT, 1% Triton X-100) using a mortar and pestle. Samples were spun 10 minutes at 15,000g and 3x SDS loading buffer (30% glycerol, 3% SDS, 94 mM Tris pH 6.8, 75mM DTT) was added to the supernatant. For anti-Ubiquitin immunoblots, total protein extracts were separated by 10% SDS-PAGE, blocked with 5% high grade BSA or 2% gelatine for 1 hour and subsequently probed with anti-Ubiquitin antibody (Figure 3.3.5, figure 3.3.6, supplemental figure 7.4A, Dako, Z0458; Supplemental figure 7.4B, Agrisea, AS08 307; 0.02% sodium azide) followed by anti-Rabbit-HRP conjugated antibody (Promega, W4028). For anti-NBR1 immunoblots, total protein extracts were separated in 10% SDS or 12% 6M urea gels by SDS- PAGE, blocked with 5% milk or 5% high grade BSA for 1 hour, and subsequently probed with anti-NBR1 antibody (kindly provided by T. Johansen, Tromsø, Norway; 1% BSA, 0.02% sodium azide) followed by anti-Rabbit-HRP conjugated antibody (Promega, W4018). For anti-ATG8a immunoblots, total protein extracts were separated on 6M 12% urea gels using SDS-PAGE,
blocked with 5% milk for 1 hour, and subsequently probed with anti-ATG8a antiserum (kindly provided by Y. Ohsumi, Okazaki, Japan; 1% BSA, 0.02% sodium azide) followed by anti- Rabbit-AP conjugated antibody (Promega, S3738; 1% milk). Amido black or coomassie blue staining of the large subunit of RuBisCO serve as loading control.

5.14 Detached leaf assay

Leafs were excised and kept for 4 days in the dark under high humidity prior to photos being taken.

5.15 Trypan blue staining

Modified from Torres et al., 2002. Leafs were infiltrated with Pst DC3000 avrRpm1 at a concentration of 10^5 bacteria, and stained for trypan blue 12 hours later. Leafs were collected in eppendorf tubes, lactophenol blue was added to tubes and boiled for 3 minutes. lactophenol blue was removed and washed in chloral hydrate, left 2-6 hours, washed again and left over night.

Lactophenol blue solution: 10 ml lactic acid, 10 ml glycerol, 10 ml phenol, 10 ml water, 0.1 g trypan blue, 80 mL ethanol.

5.16 Sphingolipids analyses

Ler acd11, acd11 NahG and NahG plants were grown for four weeks prior to spraying with 100 μM BTH. Leaf material was harvested from three biological replicates for each genotype and time point, and subsequently freeze dried and weighted. Sphingolipid analyses were performed by mass spectrometry (Bielawski et al., 2009; Markham et al., 2006).
5.17 Hela cell cultures

HeLa cells were grown in 6 well plates containing DMEM supplemented with 10% FBS, 2 mM Penicillin and Streptomycin.

5.18 Hela cells immunoblotting

Protein was extracted in ice cold RIPA buffer (50 mM Tris pH 7.5 or 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, Protease Inhibitors), concentration was measured using BCA protein assay reagent (Pierce) and 15 μg protein was separated on 4-20% SDS gels (Bio-Rad, #345-0032). Immunoblotting followed normal procedures using the following antibodies: LC3B, Cell Signaling #27755; β-actin, Sigma #A2228; FLAG, Sigma #F1804.

5.19 siRNAs and transfections

siFAPP2-b, AAGCATTCTTGGCATCATGTT; siFAPP2-v (Godi et al., 2004); siScrambled, Qiagen #1022076. siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) according to manufacturers protocol.

Plasmid transfections were performed with X-tremeGENE (Roche) according to manufacturers protocol.

5.20 ie-DAP treatment of HeLa cells

HeLa cells were transfected with siRNA constructs as described above, and 24 hours later 1-100 μg/mL ie-DAP (Invivogen) was added to the media, and total RNA was extracted 24 hours later.
5.21 Total RNA extraction and cDNA synthesis of HeLa cells

Total RNA was extracted using RNeasy Plus Mini kit (QIAGEN) according to manufacturers protocol. cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad) according to manufacturers protocol.

5.22 Long-lived protein degradation assay

Cells were treated with siRNAs using RNAiMAX as described above. 24 hours after initial transfection, cells were incubated with 2mL/well 0,25 µCi/mL L-[14C]-valine supplemented RPMI culture media. 24 hours later, cells were washed with 37°C PBS, followed by a 6 hour chase in 1mL 10mM valine supplemented RPMI culture media. Wash was repeated, and replaced with 1mL 10mM valine supplemented RPMI culture media with or without 100 nM Bafilomycin A1. 4 hours later, supernatant was transferred to eppendorf tubes and 300 µL 50% TCA was added, and tubes were incubated 30 minutes at 4°C. Supernatant was spun down. In the mean time, 500 µL 0,2M KOH was added to wells, and they were allowed to dissolve for 1 hour at 4°C. 550 µL supernatant from either eppendorfs or wells were transferred to small vials. 3 mL scintillation fluid was added to each vials, and 14C counts were measured for 3 minutes.

5.23 Statistics

Statistical analyses were performed using GraphPad Prism 6. Significance was accepted at the level of \( p \leq 0.05 \). Anova was used for analyzing all assays except the sphingolipid profiles where students t-test were used, due to small sample sizes.
5.24 Hyperlinks and software

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5.25 Acknowledgements

I thank Dingzhong Tang and Kokhi Yoshimoto for the atg2 and atg2 npr1 knockout seeds, and Kokhi Yoshimoto for the atg5 and atg5 npr1 knockout seed lines. I also thank Terje Johansen for the NBR1 antibody, and Yoshinori Ohsumi for the ATG8a antiserum. In addition thanks Suksawad Vongvisuttikun for technical assistance.
6. List of references


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7. Appendix

Supplemental table 1: Analytical overview of students t-tests (two-tailed) of phyto-C1P data presented in figure 3.1.16. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; NS, not significant.

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Supplemental figure 7.1: Photos of 7 weeks old WT, atg2, mag1 and vps35 double mutants grown under short day conditions. Scale bar indicates 1 cm.

Supplemental figure 7.2: Photos of 5, 7 and 9 weeks old Col-0 WT, npr1, atg2 and atg2 npr1 plants.
Supplemental figure 7.3: Growth of avirulent *Pst* DC3000 avrRpm1 in 5 weeks old WT, *npr1*, *atg2*, *atg2 npr1* and *rpm1* plants. Plants were inoculated with $1 \times 10^8$ colony forming units (CFU) mL$^{-1}$ and log10 bacterial counts per area of leaf were plotted against 0 and 6 hours post infection (HPI). Error bars indicate standard deviation and was calculated from the mean of 3 samples per genotype. Pairwise comparisons for all means at the 6 hour time point post infection were performed using a one-way ANOVA test followed by Tukey post-hoc test. n.s., not significant. *, $P \leq 0.05$.

Supplemental figure 7.4: A) Increased exposure of the HRP-developed immunoblot of ubiquitin from 3 week old tissue from WT, *npr1*, *atg2 npr1* and *atg2* plants, shown in figure 3.3.5. Amido black staining of the large subunit of RuBisCO serve as loading control. Anti-ubiquitin antibody from Dako. B) Two different exposures of the same HRP-developed immunoblot of ubiquitin from 6 weeks old WT, *npr1*, *atg2 npr1* and *atg2* plants. Amido black staining of the large subunit of RuBisCO serve as loading control. Anti-ubiquitin antibody from Agrisera.
**Supplemental figure 7.5:** 5 weeks old Col-0 WT, *npr1*, *atg2* and *atg2 npr1* plants grown on MS plates supplemented with 5 ng/mL tunicamycin and 50 μM BTH.

**Supplemental figure 7.6:** Single seedlings of Col-0 WT, *npr1*, *atg2 npr1* and *atg2* grown 12 days on MS plates, followed by the transfer of single seedlings to MS plates supplemented with 5 ng/mL tunicamycin and 50 μM BTH, and subsequently grown for additional 16 days before photos were taken.
8. Manuscripts & publications

- **Retromer controls immunity associated cell death in Arabidopsis.**
  David Munch, Qinsong Liu, Frederikke Gros Malinovsky, Ramesh R. Vetukuri, Peter Brodersen, Ikuko Hara-Nishimura, Jeffery L. Dangl, Morten Petersen, John Mundy, and Daniel Hofius. Awaiting final figures for re-submission to Plant Cell.

- **Autophagy deficiency leads to accumulation of ubiquitinated proteins, ER stress and cell death in Arabidopsis.**

- **Arabidopsis accelerated-cell-death11, ACD11, is a ceramide-1-phosphate transfer protein and intermediary regulator of phytoceramide levels.**

- **Role of autophagy in disease resistance and hypersensitive response-associated cell death.**
Retromer contributes to immunity associated cell death in Arabidopsis

Running title: Retromer functions in plant immunity

David Munch, a Frederikke Gro Malinovsky, a,1,2 Qinsong Liu, b,1 Ramesh R. Vetukuri, b Farid El Kasmi, c Peter Brodersen, a Ikuko Hara-Nishimura, d Jeffery L. Dangl, c Morten Petersen, a John Mundy, a and Daniel Hofius a,b,3

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Estimated length: 11.6 pages
ABSTRACT

Plants utilize nucleotide binding leucine-rich repeat (NB-LRR) immune receptors to perceive pathogen effectors, resulting in effector-triggered immunity (ETI). ETI often involves a localized programmed cell death (PCD) reaction, known as the hypersensitive response (HR), which can engage vacuole-mediated processes including autophagy. The lethal Arabidopsis mutant accelerated cell death 11 (acd11) exhibits constitutive activation of immune responses and PCD, and was previously used to screen for lasarus (laz) suppressors. The identification of laz5, encoding a Toll-interleukin-1 receptor (TIR)-NB-LRR protein, in this screen indicated that PCD in acd11 is caused by inappropriate HR activation. Other suppressors may therefore define genes required for NB-LRR function and ETI. Here, we report that laz4 is mutated in one of three Arabidopsis VPS35 genes (VPS35B, At1g75850). VPS35 is a core component of the retromer complex, which functions in endosomal trafficking and retrieval of vacuolar sorting receptors. We demonstrate that VPS35 homologs contribute to TIR- and coiled-coil (CC)-NB-LRR mediated autoimmunity and pathogen-triggered HR. Furthermore, we show that retromer deficiency attenuates disease resistance conditioned by a TIR-type NB-LRR protein, and strongly impairs autophagy-associated vacuolar processes. Our findings indicate important roles of retromer-mediated trafficking during ETI, which may include targeting of vacuolar cargo required for PCD execution.
INTRODUCTION

Programmed cell death (PCD) plays a central role in many plant processes, most notably during development and innate immunity (Hofius et al., 2007; Bozhkov and Lam, 2011; Coll et al., 2011). The plant innate immune system includes a repertoire of immune receptors that recognize different pathogens and initiate various defense responses required for disease resistance (Jones and Dangl, 2006). Surface pattern recognition receptors (PRRs) sense evolutionarily conserved pathogen-associated molecular patterns (PAMPs) and trigger antimicrobial responses known as PAMP-triggered immunity (PTI) (Schwessinger and Ronald, 2012). Intracellular immune receptors monitor the presence or activity of pathogen-derived effector proteins whose activities suppress or evade PTI (Bent and Mackey, 2007; Coll et al., 2011; Maekawa et al., 2011). Such effector-triggered immunity (ETI) often includes rapid, localized host cell death at the infection side in a process called the “hypersensitive response” (HR), which is believed to prevent the growth and spread of biotrophic pathogens (Spoel and Dong, 2012). While some evidence supports this notion (Wang et al., 2011), other examples indicate that ETI can be separated from the HR, at least in some plant-pathogen interactions (Bendahmane et al., 1999; Bulgarelli et al., 2010; Coll et al., 2010; Heidrich et al., 2011).

Most immune receptors controlling ETI belong to the family of NB-LRR proteins, named after their central nucleotide-binding (NB) and C-terminal leucine-rich repeat (LRR) domains (Caplan et al., 2008). The N-terminal regions include either a Toll/Interleukin-1 Receptor homology (TIR) or a predicted coiled-coil (CC) domain. The molecular mechanisms that regulate and execute ETI downstream of NB-LRR activation are not well known, and diversity in signaling mechanisms is likely (Eitas et al., 2008; Bonardi et al., 2011; Bonardi and Dangl, 2012). In particular, mechanisms of ETI associated PCD remain ill defined. In general, there is evidence that plants engage multiple routes to cellular demise in response to developmental and environmental cues (Bozhkov and Lam, 2011). A recent attempt to define these types of cell death focused on morphological criteria and proposed a classification into vacuolar cell death including autophagic mechanisms (see below), and necrosis (van Doorn et al., 2011). Importantly, plant HR could not be assigned to either type, since most cell death-associated morphologies show rather mixed and atypical features (van Doorn et al., 2011).

In this context, we showed that autophagy components have death-promoting functions in HR (Hofius et al., 2009). Autophagy is an evolutionarily conserved vesicular pathway for degradation and recycling of cell contents in eukaryotes, and has been
implicated in plant development, stress tolerance and pathogen defence (Liu and Bassham, 2011). Using loss-of-function mutants of Arabidopsis autophagy (ATG) genes, we provided genetic evidence that HR conditioned by activated TIR-NB-LRR proteins (i.e. RPS4, RPP1) depends on autophagy processes (Hofius et al., 2009). Autophagy components also contribute to HR triggered by the CC-NB-LRR protein RPM1 (Hofius et al., 2009; Pajerowska-Mukhtar and Dong, 2009). Consistent with this, the proteasome subunit PBA1 was shown to exhibit caspase-3-like activity and to be required for vacuole-mediated HR triggered by RPM1 and by the additional CC-NB-LRR protein RPS2 (Hatsugai et al., 2009). In contrast, caspase-1-like activity of the vacuolar protease VPE is dispensable for RPM1-triggered HR but essential to virus-induced HR conditioned by the TIR-NB-LRR N protein (Hatsugai et al., 2004; Hatsugai et al., 2009). Collectively, these data indicate that HR driven by activation of TIR- and CC-NB-LRR immune receptors may be differentiated both at the signaling and execution stages.

Mutants expressing autoimmunity- and PCD-related phenotypes are excellent genetic models to identify components of defence and cell death pathways (Moeder and Yoshioka, 2008; Palma et al., 2010; Bonardi et al., 2011). One of these is the recessive, lethal accelerated cell death 11 (acd11) Arabidopsis mutant which exhibits constitutive activation of immune responses and PCD due to disruption of a sphingolipid transfer protein (Brodersen et al., 2002; Brodersen et al., 2005). PCD in acd11 is initiated in seedlings at the 2-4 leaf stage and is dependent upon isochorismate-derived compounds including the phytohormone salicylic acid (SA). Such signaling compounds are metabolized in planta upon transgenic expression of the bacterial enzyme NahG (Heck et al., 2003; Brodersen et al., 2005), leading to full suppression of the lethal phenotype of acd11. In acd11 nahG, cell death is triggered upon application of the SA agonist benzothiadiazole-S-methyl ester (BTH). We previously reported on the use of this property to isolate an extensive series of acd11 suppressor mutants, termed lazarus (Malinovsky et al., 2010). These analyses showed that lethality in acd11 fully depends on the RPS4-like immune receptor LAZ5, and that LAZ2, a histone H3 lysine 36 methyltransferase, is required for LAZ5 expression. This indicated that loss of ACD11 results in inappropriate LAZ5 activation in the absence of pathogen effector recognition. Similarly, the PHOENIX21 CC-NB-LRR protein is required for autoimmunity in the lesion simulating disease resistance 1 (lsd1) mutant (Bonardi et al., 2009). These results indicate that suppressors of autoimmune mutants define genes required for NB-LRR protein functions.
Here we report that LAZ4 encodes one of three Arabidopsis VPS35 proteins, which are core components of retromer complexes. Plant retromer functions in the recycling of vacuolar sorting receptors (VSRs) and plasma membrane proteins during development, but has not been implicated in plant immunity before (Reyes et al., 2011; Robinson et al., 2012). We demonstrate that VPS35 genes contribute to TIR- and CC-NB-LRR protein mediated autoimmunity and HR cell death. We also demonstrate the specific involvement of retromer in disease resistance conditioned by a TIR-NB-LRR immune receptor. Finally, we provide evidence that retromer mutants are defective in HR-associated autophagic degradation, suggesting important functions of the retromer complex in vacuolar PCD.

RESULTS

Identification of LAZ4/VPS35B

laz4-1 was isolated as an ethyl-methanesulfonate (EMS)-induced, recessive suppressor of cell death in response to BTH in the Ler acd11-1 nahG background. Suppression appeared relatively weak compared to previously characterized lazarus mutants (Malinovsky et al., 2010; Palma et al., 2010), as cell death symptoms were visible in older leaf stages of laz4-1 acd11-1 nahG plants 5 days post BTH treatment (Figure 1A). However, cell death was more strongly attenuated in newly emerging leaves, which allowed laz4 acd11-1 nahG plants to survive throughout development to flower and set seed, in marked contrast to acd11-1 nahG plants (Supplemental Figure 1).

To identify the LAZ4 locus, we initially mapped laz4 to a 65 kb interval at the bottom of chromosome 1 (Figure 1B). Candidate genes with significantly induced expression in global transcript profiles of acd11 nahG plants compared to wild-type and nahG plants upon BTH treatment were sequenced (Malinovsky et al., 2010). This identified a G to A transition in a splice acceptor site of the gene At1g75850, which is annotated as VACUOLAR PROTEIN SORTING 35B (Jaillais et al., 2007) encoding one of three VPS35 homologs in Arabidopsis (Figure 1B). VPS35 proteins are highly conserved in eukaryotes and form the large subunit of the retromer complex together with VPS26 and VPS29 (McGough and Cullen, 2011). Analysis of VPS35B transcripts in laz4 further revealed that disruption of the intron splice site caused an in-frame 123 bp deletion corresponding to the loss of exon 8 (Supplemental Figure 2). Since only a single laz4 allele was identified, we introduced a 7.6 kb genomic fragment of the wild-type
LAZ4/VPS35B locus containing 1.5 kb of the predicted promoter, the transcribed region, and 300 bp of 3’ untranslated region (UTR) into laz4-1 acd11-1 nahG to test for transgenic complementation. BTH treatment of several independent T3 lines revealed growth arrest and cell death as for the parental acd11-1 nahG line in Ler (Supplemental Figure 1). In addition, introducing independent Col-0 ecotype knockout alleles of VPS35B into Col-0 acd11-2 nahG led to suppression of cell death upon BTH treatment. However, the effect of vps35b-1, a previously described Col-0 T-DNA insertion in intron 16 [the original designation vps35a-1 (Yamazaki et al., 2008) was changed to vps35b-1 according to TAIR nomenclature, see Methods] was considerably weaker compared to the Col-0 vps35b-2 (insertion in exon 9) (Figure 1C). Indeed, quantitative RT-PCR verified that vps35b-2 is a null mutant, whereas vps35b-1 still accumulated residual levels of VPS35B transcripts (Supplemental Figure 3).

Together, these results demonstrate that laz4-mediated suppression of BTH-inducible PCD in acd11 nahG is caused by a mutation in the retromer component VPS35B and we refer to it as such hereafter.

Genetic functions of VPS35B in autoimmunity-triggered PCD

We were unable to obtain surviving acd11 vps35b double mutants in the absence of nahG in both the Ler and Col-0 backgrounds (Supplemental Figure 4). This indicated either a weak suppressor activity of mutated VPS35B, or functional redundancy among the three VPS35 homologs. Indeed, previously described single knockouts of VPS35A (At2g17790) and VPS35C (At3g51310) [vps35a-1 and vps35c-1, designated according to TAIR nomenclature] (Yamazaki et al., 2008)] showed no major effects on constitutive (Supplemental Figure 4) or BTH inducible acd11-related death (Figure 1C). However, their respective combinations with the vps35b-1 allele permitted acd11-2 plants to survive in the absence of nahG (Figure. 2A, Supplemental Figure 5A). Mutant growth under lower temperature (17°C) further enhanced the suppression phenotype and allowed reproductive development and seed production (Figure. 2A). In contrast, cell death in acd11 was not suppressed in acd11-2 vps35a-1 vps35c-1 triple mutants (Supplemental Figure 5A), indicating that VPS35B is predominantly required for cell death execution in acd11. Since triple mutant combinations of all VPS35 genes could previously only be generated in the presence of a leaky loss-of-function allele of VPS35A [At2g17790; vps35a-2, designated according to TAIR (Yamazaki et al., 2008)], further genetic analysis
of VPS35 contribution to cell death in *acd11* is prevented by the overlapping and essential roles of retromer in plant viability and development.

*acd11* plants carrying a null allele of *LAZ5* due to a T-DNA insertion (*acd11-2 laz5-1* (Palma et al., 2010)) exhibited wild-type like growth under short-day conditions, but developed a distinct secondary cell death phenotype when transferred to a long-day photoperiod (Figure 2B). We speculate that this may be due to activation of an as yet uncharacterized NB-LRR protein, particularly since dominant-negative *laz5* mutants fully suppress *acd11* phenotypes (Palma et al., 2010). Notably, introducing the single *vps35b-1* T-DNA mutation into *acd11-2 laz5-1* considerably improved growth and reproductive performance under long-day conditions (Figure 2B). This indicates that VPS35B also contributes to *LAZ5*-independent forms of autoimmune cell death in *acd11*. 

To analyze whether VPS35 functions in PCD control are limited to *acd11*, we introduced the *VPS35* double mutant alleles into the lesion mimic mutant *lsd1*. Run-away cell death conditioned by the CC-NB-LRR PHOENIX21 (Bonardi et al., 2011) was strongly suppressed by *vps35b-1 c-1* but not by other mutant combinations (Figure 2C, Supplemental Figure 5B). Collectively, these findings reveal that specific VPS35 proteins have important genetic functions in PCD triggered by both TIR-type and CC-type NB-LRR immune receptors.

**VPS35 homologs function in effector-triggered HR and immunity**

Since HR-like PCD in *acd11* and *lsd1* is mediated by inappropriate activation of NB-LRR immune receptors, suppressors of PCD may represent important regulators of NB-LRR proteins. Thus, VPS35B and homologous VPS35 proteins may also be required for HR activated upon recognition of pathogen effectors. To investigate this, *vps35* double mutant combinations were challenged with *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 strains expressing specific effectors to trigger ETI. In addition to the previously described double mutants *vps35a-1 c-1* and *vps35b-1 c-1* (Yamazaki et al., 2008), we generated *vps35a-1 b-1* in the absence of *acd11*. *vps35a-1 b-1* showed a similar dwarfing phenotype as *vps35a-1 c-1* but did not exhibit early leaf senescence (Supplemental Figure 6). To avoid potential interference of age-related processes with effects on HR and disease resistance measured by pathogen growth restriction, experiments were performed on leaves of short-day grown plants (5-6 weeks) prior to the onset of senescence (Hofius et al., 2009; Hofius et al., 2011). HR was monitored by ion leakage assays, as conductance...
increases upon electrolyte release from dying leaf tissue (Mackey et al., 2003; Hofius et al., 2009). These assays showed that the double mutants displayed marked differences in HR depending on the mutant combination and ETI event (Figure 3). Most strikingly, vps35a-1 c-1 showed consistent and strong suppression of ETI triggered upon recognition of AvrRps4, AvrRpm1 and AvrRpt2 effector proteins. AvrRpm1- and AvrRpt2-induced electrolyte leakage in vps35a-1 c-1 was higher than that in the respective rpm1-3 and rps2-101C controls (Figures 3B and 3C). AvrRps4-triggered electrolyte leakage was dramatically lower in vps35a-1 c-1 than in rps4-2 (Figure 3A). This suggested that cell death triggered by RPS4-independent recognition of AvrRps4 in Col-0 was also suppressed. Reductions in ion leakage observable in other vps35 mutant combinations were less consistent than in vps35a-1 c-1, and could only be supported by statistical significance for AvrRpm1- (P<0.05 at 6 hpi, Figure 3B) and AvrRpt2-triggered HR (P<0.05 at 19 hpi, Figure 3C) in vps35b-1 c-1, as well as for early time points (P<0.01 at 7-11 hpi, Figure 3C) of RPS2-conditioned HR cell death in vps35a-1 b-1.

To further analyze whether the observed changes in HR resulted in altered disease resistance, bacterial titers were determined (Figure 4). Notably, reduced levels of cell death triggered upon recognition of AvrRps4 were accompanied by up to 14-fold enhanced bacterial growth in vps35a-1 c-1 double mutants (Figure 4A). In contrast, RPM1- and RPS2-conditioned resistance remained largely unaffected, even in association with severe HR suppression in vps35a-1 c-1 (Figures 4B and 4C). Overall, these results indicate that VPS35A and VPS35C function in disease resistance conditioned by a TIR-NB-LRR protein, and contribute to HR mediated by two CC-NB-LRR proteins.

Finally, to assess whether VPS35 homologs are also required for basal defense, vps35 double mutants were infected with virulent Pst DC3000. As indicated in Figure 4D, bacterial growth in the different vps35 mutant combinations was indistinguishable from wild-type controls. This suggests that VPS35 deficiency does not compromise the induction of basal defence responses upon bacterial infection.

**Retromer dysfunction suppresses HR**

Suppression of HR by VPS35 loss-of-function mutations implicates VPS35-containing retromer complexes in HR. We therefore assayed whether disruption of another core retromer component similarly affects HR and disease resistance. VPS29 is encoded by a single gene in Arabidopsis and vps29 null alleles display severe dwarfism and
morphological changes which may interfere with analyses of immune system function. Therefore, we used the leaky T-DNA allele mag1-1 (Shimada et al., 2006), which showed only moderate growth reduction and phenotypically resembled the vps35a-1 b-1 double mutant (Supplemental Figure 6). Ion leakage assays following infection with Pto DC3000 expressing AvrRps4 or AvrRpt2 indicated that mag1-1 mutants were also compromised in RPS4- and RPS2-conditioned HR (Figures 5A and 5B). Consistent with this, and similar to the vps35a-1 c-1 double mutant, mag1-1 supported increased growth of AvrRps4 expressing Pst DC3000 (P<0.0001, Figure 5C), Together, these results indicate that dysfunction of the retromer complex, as a result of reduced VPS35 or VPS29 levels, is responsible for suppression of HR, as well as for partial abrogation of bacterial disease resistance conditioned a TIR-NB-LRR immune receptor. 

Retromer mutants are impaired in autophagy processes
Retromer-dependent processes are implicated in the trafficking of vacuolar proteins, which may be required for late steps of HR (Hara-Nishimura and Hatugai, 2011; Robinson et al., 2012). Since autophagy contributes to vacuole-mediated forms of cell death conditioned by RPS4 and RPM1 (Hofius et al., 2009), we speculated that interruption of vacuolar trafficking in retromer mutants may be revealed by defects in autophagy. To test this, we first compared retromer with autophagy deficient (atg) mutants in their response to nutrient limitation. Similar to atg2-1 and atg7-2, vps35a-1 c-1 and mag1-1 mutants responded with exaggerated senescence to leaf detachment and prolonged darkness, whereas vps35a-1 b-1 and vps35b-1 c-1 remained largely unaffected (Figure 6A). To further investigate whether autophagic activity is affected in retromer mutants, we monitored protein levels of the autophagy adaptor protein NBR1, whose vacuolar degradation is a marker for autophagic flux (Svenning et al., 2011). As expected, NBR1 levels remained low in untreated wild-type seedlings due to basal autophagy activity, but were strongly elevated in atg2-1. Importantly, vps35a-1 b-1, vps35a-1 c-1 and mag1-1 showed significant enhancement of NBR1 compared to wild-type, indicating that autophagic degradation cannot be completed in some retromer mutants (Figure 6B).

Finally, we analyzed the impact of retromer dysfunction on HR-associated autophagy, which is rapidly and strongly induced by Pst DC3000 expressing AvrRpm1 (Hofius et al., 2009). NBR1 levels in wild-type and vps35b-1 c-1 showed a characteristic biphasic response until 6 hours post infection (hpi). In contrast, vps35a-1 b-1, vps35a-1 c-
and mag1-1 mutants were clearly defective in RPM1-dependent autophagic flux (Figure 6C). In particular, vps35a-1 c-1 plants displayed a dramatic increase of NBR1 protein during infection resulting in comparable levels as in the constitutively NBR1 accumulating atg2-1 mutant. Overall, these findings indicate that certain retromer mutants are defective in vacuolar processes required for execution of HR death-promoting autophagy.

**DISCUSSION**

**A novel role for retromer in immune receptor-mediated HR**

Retromer is a multi-subunit complex consisting of the cargo recognition VPS35-VPS29-VPS26 heterodimer and the membrane-targeting subcomplex of sorting nexins (SNXs) (Attar and Cullen, 2010). Its best-described function in various organisms is the recycling and retrograde transport of vacuolar or lysosomal acid hydrolase receptors from endosomes back to the trans-Golgi network (TGN) (McGough and Cullen, 2011; Robinson et al., 2012). In addition, retromer-mediated endosomal trafficking and retrieval has been shown for a range of other transmembrane receptors, and revealed retromer roles in processes such as Wnt-dependent signaling, apoptotic cell clearance, and the development of Alzheimer’s diseases (Port et al., 2008; Chen et al., 2010; Lane et al., 2010). In plants, retromer has been implicated in recycling vacuolar sorting receptors (VSRs) for efficient delivery of seed storage proteins, and in trafficking PIN efflux carriers involved in polar auxin transport (Oliviusson et al., 2006; Kleine-Vehn et al., 2008; Kang et al., 2012). These findings were based on analyses of loss-of-function mutants of SNXs and core retromer components which exhibited multiple developmental phenotypes related to embryogenesis, organogenesis, cell polarity, and leaf senescence (Jaillais et al., 2007; Yamazaki et al., 2008; Pourcher et al., 2010).

We provide evidence that the retromer complex is also required for immunity-related processes. We found that laz4, which partly suppresses PCD in acd11, encodes the retromer component VPS35B. VPS35B is one of three Arabidopsis VPS35 homologs which we show have important genetic functions in the control of immune receptor mediated PCD. VPS35B seems to play a predominant role in PCD triggered by the TIR-NB-LRR protein LAZ5, since loss-of-function mutations in VPS35B, but not in VPS35A or VPS35C, suppressed BTH-induced cell death in acd11 nahG (Figure 1). However, VPS35A and VPS35C can partially substitute the genetic function of VPS35B, as
constitutive acd11 cell death in the absence of nahG was attenuated only by vps35b-1 in combination with either vps35a-1 or vps35c-1 knockouts (Figure 2A). When grown under moderately reduced temperature (17°C), the mutants exhibited an enhanced suppressive phenotype. This may be due to an additional block of endosomal and/or retromer associated trafficking (Kuismanen and Saraste, 1989; Gentzsch et al., 2004). Importantly, VPS35B function in acd11 PCD is not restricted to LAZ5-conditioned cell death, but also applies to PCD pathways activated under long-day conditions by potentially (an)other, yet unknown immune receptor(s) (Figure 2A). When grown under moderately reduced temperature (17°C), the mutants exhibited an enhanced suppressive phenotype. This may be due to an additional block of endosomal and/or retromer associated trafficking (Kuismanen and Saraste, 1989; Gentzsch et al., 2004). Importantly, VPS35B function in acd11 PCD is not restricted to LAZ5-conditioned cell death, but also applies to PCD pathways activated under long-day conditions by potentially (an)other, yet unknown immune receptor(s) (Figure 2A). In support of this notion, the remarkable suppression of lsd1 cell death by combined loss-of-function mutations in VPS35B and VPS35C reveal that distinct VPS35 proteins can also contribute to autoimmunity activated by a CC-NB-LRR protein. This suggests a more general role of VPS35-dependent processes in TIR- and CC-NB-LRR triggered autoimmune cell death, and implies that the other R protein(s) involved in Col-0 acd11 cell death may belong to the CC-NB-LRR class.

Although vps35a-1 vps35c-1 double mutants did not suppress autoimmunity-triggered PCD (Supplemental Figure 4), they strongly impacted HR cell death conditioned by RPM1 and RPS2 (Figures 3B and 3C) and ETI conditioned by RPS4 (Figure 3A). This suggests remarkable variation in the contribution of individual VPS35 proteins to plant immunity. Similar functional divergence between the three VPS35 homologs has been observed in plant development, as loss-of-function of VPS35A, but not of VPS35B or VPS35C, suppressed the gravitropic and morphological abnormalities of the zip1/vti11 mutant (Hashiguchi et al., 2010). Nonetheless, vps35c-1 and to a lesser extent vps35b-1 were able to enhance the suppressive effect of vps35a-1, supporting the notion that VPS35 genes still share some common functions. As proposed earlier, the acquisition of complex functional specialization among VPS35 family members may be reflected in their cargo selection, subcellular localization, or assembly into different retromer combinations (Hashiguchi et al., 2010). Such isoform-specific variations in retromer composition are probably also dependent on plant tissue, developmental stage and environmental conditions (Jaillais et al., 2007; Pourcher et al., 2010). For instance, mass spectrometric analysis of VPS29 immunocomplexes in untreated Arabidopsis leaves detected VPS35A and VPS35C but not VPS35B (Jaillais et al., 2007), which may indicate a less important role of VPS35B during normal development. However, more systemic
biochemical investigations of retromer subcomplexes and associated cargo are needed to clarify the recruitment of individual VPS35 proteins in different biological contexts.

Our additional analysis of ETI responses in the mag1-1 mutant provides evidence that disruption of retromer function is responsible for the impact on immunity-associated PCD. Depletion of the core component VPS29, encoded by a single gene, has previously been shown to destabilize and reduce VPS35 protein levels, indicating that these proteins function together in the retromer complex (Shimada et al., 2006). Although analyses of immune responses could not be applied to severely dwarfed vps29 null mutants (Shimada et al., 2006; Jaillais et al., 2007), reduced revels of the retromer core complex in the weak mag1-1 allele attenuated HR in response to Pst DC3000 AvrRps4 and AvrRpt2, respectively (Figure 5). However, the suppressive effect in mag1-1 was less pronounced than in vps35a-1 c-1 doubles, which is probably due to the incomplete block of VPS29 expression in mag1-1. Overall, due to the phenotypic constraints or lethality of true retromer null mutants (Jaillais et al., 2007; Yamazaki et al., 2008), it is tempting to assume that our data underestimate the actual contribution of retromer functions to cell death execution.

Distinct functions of retromer in TIR-NB-LRR activated disease resistance

Suppression of cell death conditioned by the CC-NB-LRR proteins RPM1 and RPS2 in retromer mutants was not accompanied by changes in bacterial growth restriction (Figures 4B and 4C). This supports the increasing evidence that NB-LRR conditioned disease resistance can be uncoupled from HR (Bendahmane et al., 1999; Coll et al., 2010; Heidrich et al., 2011). However, during RPS4 activated immunity, both cell death and pathogen growth restriction were compromised in vps35a-1 c-1 and mag1-1 (Figures 4A and 5C). These data suggest additional and distinct roles of retromer in immune responses activated by a TIR-NB-LRR. RPS4-dependent HR is correlated with cytosolic RPS4-EDS1 complexes, while disease resistance requires nuclear accumulation of AvrRps4, RPS4 and EDS1 (Wirthmueller et al., 2007; Heidrich et al., 2011). Therefore, the suppression of RPS4-mediated HR and disease resistance in retromer deficient backgrounds may place retromer components upstream of HR and nuclear defence activation, and might indicate altered trafficking and compartmentalization of the effector or NB-LRR protein. However, recent characterization of AvrRps4 mutant alleles revealed uncoupling of avirulence and cell death without changes in subcellular localization (Sohn
et al., 2012). This suggests that different thresholds are required in AvrRps4-mediated disease resistance and HR (Robinson et al., 2012). In addition, there is emerging evidence for the complex involvement of several TIR-NB-LRR proteins in AvrRps4-triggered ETI (Bonardi and Dangl, 2012; Sohn et al., 2012), which may account for the proposed dose-dependent defense activation. Our observation of significantly stronger suppression of cell death in vps35a-1 c-1 and vps29/mag1-1 compared to the rps4-2 knockout line suggests that retromer contributes to at least some of the RPS4-independent responses.

**Vacuolar trafficking during HR cell death execution**

Our data suggest a general role of retromer-associated processes in HR. Additionally, our data suggest distinct functions of the retromer complex in limiting pathogen growth during TIR-NB-LRR mediated ETI. A common theme in HR is the requirement of vacuole-mediated execution steps (Hara-Nishimura and Hatsugai, 2011). RPS2-dependent HR involves fusion of the tonoplast with the plasma membrane and discharge of vacuolar hydrolytic enzymes into the extracellular matrix (Hatsugai et al., 2009). RPS4-dependent HR engages autophagy mechanisms (Hofius et al., 2009), which require intravacuolar breakdown of autophagosomes and their transported cargo. Late steps of autophagy-dependent cell death may also involve collapse of vacuolar membranes and release of hydrolytic enzymes into the cytosol (van Doorn et al., 2011). Interestingly, both forms of vacuolar cell death seem to be induced upon RPM1 activation (Hatsugai et al., 2009; Hofius et al., 2009). The marked accumulation of the selective autophagy substrate NBR1 in vps35a-1 c-1 following RPM1 activation (Figure 6C) strongly supports the view that retromer deficiency interferes with vacuolar processes, and thus impairs autophagic flux during the HR. Due to the essential role of retromer in recycling of VSRs, it is expected that retromer dysfunction broadly disrupts intracellular trafficking of vacuolar cargo (Oliviousson et al., 2006; Yamazaki et al., 2008; Kang et al., 2012). Therefore, execution steps of autophagy and vacuole-fusion mediated cell death pathways may likewise be affected, which could explain the severe suppression of HR mediated by both TIR- and CC-NB-LRR proteins in vps35a-1 c-1 mutants.

Our observations that retromer mutants exhibit additional defects in basal and starvation-induced autophagy (Figures 6A and 6B) further implicate important functions of retromer components and trafficking in autophagic mechanisms. In this regard, it is noteworthy that a recent proteomics analysis of autophagosome composition in
mammalian cell cultures identified VPS35 as an associated protein (Dengjel et al., 2012). Subsequent genetic studies of the yeast orthologues revealed that \textit{vps35} knockouts are as defective in autophagy as \textit{atg} mutants (Dengjel et al., 2012). Furthermore, a human Rab GTPase activating protein (GAP) interacted with the retromer component VPS29 and the autophagy protein ATG8/LC3, and was suggested to function as molecular switch between retromer-decorated endosomes and autophagosomes (Popovic et al., 2012). Future investigations may reveal whether retromer subunits are also associated with autophagy components or compartments in plants.

In conclusion, this study provides a primary example of the involvement of retromer components in HR and disease resistance, and highlights the emerging importance of specific membrane trafficking routes in ETI (Nomura et al., 2011). Our finding that the retromer complex is genetically linked to NB-LRR mediated HR signaling that also engages autophagy and/or other vacuole-mediated processes suggests a complex interrelationship between endosomal, autophagic, and vacuolar trafficking events. Our genetic models of autoimmunity- and pathogen-triggered HR are valuable tools to further dissect these pathways and characterize their regulatory interactions.

\section*{METHODS}
\subsection*{Plant material and growth conditions}
Arabidopsis \textit{acd11} and \textit{acd11 nahG} in \textit{Ler (acd11-1)} and \textit{Col-0 (acd11-2)}, as well as \textit{Col-0 nahG, lsd1-2, eds1-2 (Ler eds1-2 introgressed into Col-0), ndr1-1, rps4-2, rpm1-3, rps2-101c, acd11-2 laz5-1} have been described (Mindrinos et al., 1994; Aarts et al., 1998; Boyes et al., 1998; Brodersen et al., 2005; Bartsch et al., 2006; Kaminaka et al., 2006; Wirthmueller et al., 2007; Palma et al., 2010). The autophagy (\textit{atg}) deficient T-DNA insertion mutants \textit{atg7-2} and \textit{atg2-1} were described before (Inoue et al., 2006; Hofius et al., 2009). \textit{Col-0 vps35a-1, vps35b-1, vps35c-1,} and \textit{magl-1} single as well as \textit{vps35a-1 c-1} and \textit{vps35b-1 c-1} double mutants were characterized previously (Shimada et al., 2006; Yamazaki et al., 2008), and \textit{vps35a-1} and \textit{vps35b-1} mutant alleles were designated according to The Arabidopsis Information Resource (TAIR, www.arabidopsis.org) nomenclature for \textit{VPS35A} (At2g17790) and \textit{VPS35B} (At1g75850). The \textit{vps35b-2} T-DNA insertion line (GK-784C05) was obtained from the Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.info, Nottingham, UK), and plants homozygous for the insertion were verified with T-DNA left border and gene specific primers (5'-
TTATGATTCAAGTATCAAACAGCCA-3’ and 5’-CCCTGGACGTGAATGTAGACAC-3’). Sequences of primers used to select the different mutant alleles in genetic crosses are available upon request.

Following seed surface sterilization and treatment at 4°C for 2 d, plants were grown in soil under short-day conditions (8/16 h light/dark cycles) in growth cabinets, and under long-day conditions (16/8 h light/dark cycles) in a growth room at 150 μE/m²s, 21°C and approx. 70% relative humidity. Sterile plants were grown on MS agar plates with an 8 or 12 h photoperiod. The mutant screen and growth of F2 mapping populations were performed under controlled greenhouse conditions as described (Malinovsky et al., 2010; Palma et al., 2010).

Map-based cloning of the LAZ4 locus

Ler laz4-1 acd11-1 nahG was isolated as BTH-resistant suppressor in a M2 population of EMS-mutagenized acd11 nahG seeds (Malinovsky et al., 2010; Palma et al., 2010), and crossed with Col-0 acd11-2 nahG to generate a mapping population. Rough mapping was initiated on 30-40 F2 plants homozygous for laz4 using standard SSLP markers (Zhang et al., 2007), and fine-mapping was performed on 698 F2 plants with SSLP and CAPS markers designed from the Arabidopsis polymorphism and Landsberg sequence collection (http://www.arabidopsis.org/browse/Cereon/index.jsp). This mapped the LAZ4 locus to ~65 kb on the bottom of chromosome 1. Microarray-derived expression profiles of genes in the interval between At1g75790 and At1g75970 were analyzed for increased expression in acd11 nahG relative to wild-type and nahG controls (Malinovsky et al., 2010), and candidates were sequenced.

Treatments, ion leakage and bacterial resistance assays

PCD analysis of mapping populations, complemented lines and mutant crosses were done after leaf spraying with 100 μM BTH. Ion leakage assays following syringe-infiltration of avirulent Pseudomonas syringae pv. tomato (Pst) DC3000 strains were performed with 2 x 10⁸ CFU ml⁻¹ as described (Hofius et al., 2009). Resistance assays were carried out with bacteria at OD₆₀₀ = 0.0001 (virulent strain Pst DC3000, and avirulent Pst DC3000 AvrRpm1 or AvrRpt2), and OD₆₀₀ = 0.00005 (Pst avirulent DC3000 AvrRps4) essentially as described (Mackey et al., 2003).
Immunoblot analysis

Equal amounts of seedlings or leaf material were homogenized in protein extraction buffer [4M urea, 100 mM DTT, 1% (v/v) Triton X-100] and incubated on ice for 10 min. Protein samples were mixed 1:1 (v/v) with 2x Laemmli sample buffer, boiled for 10 min and centrifuged at 13,000 rpm for 10 min. Total proteins of the supernatant were subjected to SDS-PAGE, and protein loading was verified by quantification of coomassie brilliant blue (CBB)-stained bands using Image J software. Equilibrated protein samples were separated, transferred to PVDF membranes (Amersham Hybond-P, GE Healthcare, Buckinghamshire, UK), and blocked with 5% (w/v) non-fat milk powder in PBS containing 0.05% (v/v) Tween 20 (TPBS). Anti-AtNBR1 (kindly provided by T. Johansen, Tromsø University, Norway) and secondary HRP-conjugated antibodies (Amersham, GE Healthcare) were diluted 1:2000 and 1:5000 in TPBS with 1% (w/v) milk powder, respectively. The immunoreaction was developed using ECL Prime kit (Amersham, GE Healthcare) and detected in LAS-3000 Luminescent Image Analyzer (Fujiﬁlm, Fuji Photo Film, Germany).

Statistical analysis

Statistical analysis was done using one-way Anova with post-hoc Tukey´s test. Significance was accepted at the level of P<0.05.

Accession numbers:

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: VPS35A, AT2G17790; VPS35B, AT1G75850; VPS35C, AT3G1310; VPS29, AT3G47810; ACD11, AT2G34690; LSD1, AT4G20380; RPS4, AT5G45250; RPM1, AT3G07040; RPS2, AT4G26090; EDS1, AT3G48090; NDR1, AT3G20600.

SUPPLEMENTAL DATA

The following materials are available in the online version of this article.

Supplemental Figure 1. Transgenic complementation of laz4 mutant.

Supplemental Figure 2. EMS-induced intron splice mutation in laz4 results in deletion of exon 8 from VPS35B transcripts.

Supplemental Figure 3. Analysis of VPS35B transcript levels in T-DNA mutant alleles.
vps35b-1 (SALK_014345) and vps35b-2 (GABI_784C05).

**Supplemental Figure 4.** Single loss-of-function alleles in VPS35 genes do not suppress PCD in acl11-2 in the absence of nahG.

**Supplemental Figure 5.** Combined loss-of-function mutations in VPS35A and VPS35C do not suppress acl11 and lsd1 autoimmune cell death.

**Supplemental Figure 6.** Phenotypes of retromer deficient mutants.

**Supplemental Table 1.** Oligonucleotides used in Supplemental Figures 1 and 3.

**Supplemental Methods**

**Supplemental References**

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**AUTHOR CONTRIBUTIONS**


**FIGURE LEGENDS**

**Figure 1.** laz4 encodes the retromer component VPS35B.

(A) 28-day-old acl11-1 nahG, laz4-1 acl11-1 nahG, and Ler nahG plants 10 days after treatment with 100 µM BTH. Size bar = 1 cm.
(B) The *laz4* locus was mapped to a 65 kb interval between two markers on BAC T4O12 on chromosome 1. A G-to-A transition was found at the splice acceptor site of exon 8 in the *At1g75850* gene, encoding the retromer component VPS35B. Structure of the *LAZ4/VPS35B* gene (*At1g75850*) showing positions of the *laz4-1* mutation (red) as well as the T-DNA insertions (open triangles) in vps35b-1 (SALK_014345) and vps35b-2 (GABI_784C05). Closed boxes indicate exons, and lines between boxes introns.

(C) Cell death phenotype of *acd11-2 nahG* harboring vps35b-1, vps35b-2, vps35a-1 (SALK_039689), and vps35c-1 (SALK_099735 in comparison to *acd11-2 nahG* and Col-0 wild-type controls. Photos were taken 10 days after BTH treatment of 4-week-old plants. Bars indicate 1 cm.

**Figure 2.** Suppression of *acd11*- and *lsd1*-triggered autoimmunity by loss-of-function mutations in *VPS35* genes.

(A) *acd11-2*, *acd11-2 vps35a-1 vps35b-1*, and *acd11-2 vps35b-1 vps35c-1* plants grown for 4 weeks under short day (SD) conditions at 21°C or 17°C. Bars indicate 1 cm.

(B) *acd11-2 laz5-1 vps35b-1* and *acd11 laz5-1* plants grown at ambient temperatures (21°C) for 5 weeks under SD conditions followed by 2 weeks under long-day conditions.

(C) BTH-triggered run-away cell death in *lsd1-2* compared to *lsd1-2 vps35a-1 b-, lsd1-2 vps35b-1 c-1*, and Col-0 WT. Plants were grown under SD conditions for 25 days and sprayed with 100 µM BTH. Photographs were taken 8 days after treatment in comparison to untreated controls.

Scale bars indicate 1 cm in (A) and (C), and 2 cm in (B). dpa, days post application.

**Figure 3.** VPS35 proteins are required for pathogen-triggered HR cell death.

(A)-(C) Ion leakage assays of 5- to 6-week-old Col-0 WT and *vps35a-1 b-1, vps35a-1 c-1, vps35b-1 c-1* double mutants after inoculation with avirulent strains of *Pst* DC3000 expressing AvrRps4 (A), AvrRpm1 (B), or AvrRpt2 (C). Loss-of-functions mutants of the corresponding *R* genes *RPS4 (rps4-2)* in (A), *RPM1 (rpm1-3)* in (B), and *RPS2 (rps2-101C)* in (C) served as additional controls. Mean and standard errors (SE) were calculated from four disks per treatment with 3-4 replicates within an experiment.

**Figure 4.** Effect of VPS35 deficiency on plant innate immunity.
(A)-(C) Growth of avirulent stains of *Pst* DC3000 expressing *AvrRps4* in (A), *AvrRpm1* in (B) or *AvrRpt2* (C) in 6-week-old Col-0 WT and *vps35a-1 b-1*, *vps35a-1 c-1*, and *vps35b-1 c-1* double mutants 0 and 3 days post inoculation. Enhanced disease susceptibility in *eds1-1* (A), *rpm1-3* (B) and *ndr1-1* (C) served as additional control. (D) Growth of virulent strains of *Pst* DC3000 in Col-0 WT and *vps35a-1 b-1*, *vps35a-1 c-1*, *vps35b-1 c-1*, and *eds1-2* mutants 0 and 3 days after infection. Log-transformed values are means ± standard deviation (SD) with n = 3 in (A) and (B) or n = 4-6 in (C) and (D). Asterisks indicate statistical significance (P<0.001) determined by one-way Anova with post-hoc Tukey’s test (compared to wild-type). cfu: colony forming units.

**Figure 5.** Effect of VPS29 deficiency on HR cell death and disease resistance. (A)-(B) Ion leakage assays of 6-week-old Col-0 WT, *mag1-1* and *vps35a-1 c-1* plants after inoculation with avirulent strains of *Pst* DC3000 expressing *AvrRps4* (A) or *AvrRpt2* (B). Mean and standard errors (SE) were calculated from four disks per treatment with 4 (A) or 3 (B) replicates within an experiment. (C)-(D) Growth of avirulent strains of *Pst* DC3000 expressing *AvrRps4* (C) or *AvrRpt2* (D) in 6-week-old Col-0 WT, *mag1-1*, and *eds1-2* (C) or *ndr1-1* (D) 0 and 3 days post inoculation. Log-transformed values are means ± SD (n = 3). Asterisks indicate statistical significance (P<0.0001) in *mag1-1* determined by one-way Anova with post-hoc Tukey’s test (compared to wild-type). cfu: colony forming units.

**Figure 6.** Retromer is required for autophagy processes. (A) Leaf detachment assay of Col-0 WT, *atg7-2* and *atg2-1* in comparison to *vps35a-1 b-1*, *vps35a-1 c-1*, *vps35b-1 c-1* double mutants and *mag1-1*. Detached leaves of 4-week-old plants were kept for 4 days on moist filter paper in darkness. (B) Immunoblot analysis of NBR1 accumulation in 10-day-old seedlings of Col-0 WT and *atg2-1* controls in comparison to *vps35a-1 b-1*, *vps35a-1 c-1*, *vps35b-1 c-1* double mutants and *mag1-1*. Equal amounts of crude extracts were separated by SDS-PAGE and probed on blots with anti-NBR1 antibody. Comassie brilliant blue (CBB) staining of Rubisco large subunit (RbcS) served as loading control. (C) Immunoblot analysis of NBR1 accumulation upon infection with *Pst* DC3000 (*AvrRpm1*). Total proteins were extracted at the indicated time points from 5-week-old retromer mutants in comparison to Col-WT and *atg2-1* controls, and probed with ani-
NBR1 antibody (upper panels). Lower panels indicate Commassie staining of RbcS as loading control.

REFERENCES


**Figure 1.** *laz4* encodes the retromer component VPS35B.

(A) 28-day-old *acd11-1 nahG, laz4-1 acd11-1 nahG*, and *Ler nahG* plants 10 days after treatment with 100 µM BTH. Size bar = 1 cm.

(B) The *laz4* locus was mapped to a 65 kb interval between two markers on BAC T4O12 on chromosome 1. A G-to-A transition was found at the splice acceptor site of exon 8 in the *At1g75850* gene, encoding the retromer component VPS35B. Structure of the LAZ4/VPS35B gene (*At1g75850*) showing positions of the *laz4-1* mutation (red) as well as the T-DNA insertions (open triangles) in *vps35b-1* (SALK_014345) and *vps35b-2* (GABI_784C05). Closed boxes indicate exons, and lines between boxes introns.

(C) Cell death phenotype of *acd11-2 nahG* harboring *vps35b-1*, *vps35b-2*, *vps35a-1* (SALK_039689), and *vps35c-1* (SALK_099735) in comparison to *acd11-2 nahG* and Col-0 wild-type controls. Photos were taken 10 days after BTH treatment of 4-week-old plants. Bars indicate 1 cm. dpa: days post application.
Figure 2. Suppression of *acd11* and *lsd1*-triggered autoimmunity by loss-of-function mutations in *VPS35* genes.  
(A) *acd11-2, acd11-2 vps35a-1 vps35b-1*, and *acd11-2 vps35b-1 vps35c-1* plants grown for 4 weeks under short day (SD) conditions at 21°C or 17°C. Bars indicate 1 cm.  
(B) *acd11-2 laz5-1 vps35b-1* and *acd11 laz5-1* plants grown at ambient temperatures (21°C) for 5 weeks under SD conditions followed by 2 weeks under long-day conditions.  
(C) BTH-triggered run-away cell death in *lsd1-2* compared to *lsd1-2 vps35a-1 b-1, lsd1-2 vps35b-1 c-1*, and Col-0 WT. Plants were grown under SD conditions for 25 days and sprayed with 100 µM BTH. Photographs were taken 8 days after treatment in comparison to untreated controls.  
Scale bars indicate 1 cm in (A) and (C), and 2 cm in (B). dpa, days post application.
Figure 3. VPS35 proteins are required for pathogen-triggered HR cell death. 
(A)-(C) Ion leakage assays of 5- to 6-week-old Col-0 WT and vps35a-1 b-1, vps35a-1 c-1, and vps35b-1 c-1 double mutants after inoculation with avirulent strains of Pst DC3000 expressing AvrRps4 (A), AvrRpm1 (B), or AvrRpt2 (C). Loss-of-functions mutants of the corresponding R genes RPS4 (rps4-2) in (A), RPM1 (rpm1-3) in (B), and RPS2 (rps2-101C) in (C) served as additional controls. Mean and standard errors (SE) were calculated from four disks per treatment with 3-4 replicates within an experiment.
Figure 4. Effect of VPS35 deficiency on plant innate immunity. 
(A)-(C) Growth of avirulent stains of Pst DC3000 expressing AvrRps4 in (A), AvrRpm1 in (B) or AvrRpt2 (C) in 6-week-old Col-0 WT and vps35a-1 b-1, vps35a-1 c-1, and vps35b-1 c-1 double mutants 0 and 3 days post inoculation. Enhanced disease susceptibility in eds1-1 (A), rpm1-3 (B) and ndr1-1 (C) served as additional control. (D) Growth of virulent stains of Pst DC3000 in Col-0 WT and vps35a-1 b-1, vps35a-1 c-1, vps35b-1 c-1, and eds1-2 mutants 0 and 3 days after infection. Log-transformed values are means ± standard deviation (SD) with n = 3 in (A) and (B) or n = 4-6 in (C) and (D). Asterisks indicate statistical significance (P<0.001) determined by one-way Anova with post-hoc Tukey’s test (compared to WT). cfu: colony forming units.
**Figure 5.** Effect of VPS29 deficiency on HR cell death and disease resistance.  
(A)-(B) Ion leakage assays of 6-week-old Col-0 WT, mag1-1 and vps35a-1 c-1 plants after inoculation with avirulent strains of *Pst* DC3000 expressing AvrRps4 (A) or AvrRpt2 (B). Mean and standard errors (SE) were calculated from four disks per treatment with 4 (A) or 3 (B) replicates within an experiment.  
(C)-(D) Growth of avirulent strains of *Pst* DC3000 expressing AvrRps4 (C) or AvrRpt2 (D) in 6-week-old Col-0 WT, mag1-1, and eds1-2 (C) or ndr1-1 (D) 0 and 3 days post inoculation. Log-transformed values are means ± SD (n = 3). Asterisks indicate statistical significance (P<0.0001) in *mag1-1* determined by one-way Anova with post-hoc Tukey’s test (compared to wild-type). cfu: colony forming units.
**Figure 6.** Retromer is required for autophagy processes.

(A) Leaf detachment assay of Col-0 WT, *atg7-2* and *atg2-1* in comparison to *vps35a-1 b-1*, *vps35a-1 c-1*, *vps35b-1 c-1* double mutants and *mag1-1*. Detached leaves of 4-week-old plants were kept for 4 days on moist filter paper in darkness.

(B) Immunoblot analysis of NBR1 accumulation in 10-day-old seedlings of Col-0 WT and *atg2-1* controls in comparison to *vps35a-1 b-1*, *vps35a-1 c-1*, *vps35b-1 c-1* double mutants and *mag1-1*. Equal amounts of crude extracts were separated by SDS-PAGE and probed on blots with anti-NBR1 antibody. Commassie brilliant blue (CBB) staining of Rubisco large subunit (*RbcS*) served as loading control.

(C) Immunoblot analysis of NBR1 accumulation upon infection with *Pst* DC3000 (*AvrRpm1*). Total proteins were extracted at the indicated time points from 5-week-old retromer mutants in comparison to Col-WT and *atg2-1* controls, and probed with anti-NBR1 antibody (upper panels). Lower panels indicate Commassie staining of RbcS as loading control.
Supplemental Figure 1. Transgenic complementation of laz4 mutant.
Ler laz4-1 acd11 nahG plants were transformed with a 7.6 kb genomic fragment of the wild-type LAZ4/VPS35B locus containing 1.5 kb of the predicted promoter, the transcribed region, and 300 bp of 3’ untranslated region. T3 progenies of several independent transgenic lines were selected on MS medium, transferred to soil and treated with 100 µM BTH (4 times every 2nd day).

(A) Rosette phenotypes of 34-day-old acd11 nahG, laz4 acd11 nahG (2 plants each), and six transgenic lines expressing pVPS35B:VPS35B in laz4 acd11 nahG backgrounds 10 days after BTH treatment. Bar indicates 1 cm.

(B) Reproductive development is restricted to laz4 acd11 nahG plants and inhibited by cell death activation in acd11 nahG and pVPS35B:VPS35B expressing laz4 acd11 nahG lines. Photos are of 38-day-old plants 14 days after BTH treatment. dpa, days post application.
Munch et al., Supplemental Figure 2

(A) Exon-intron structure of VPS35B showing positions of EMS-induced G to A transition in las4 and exon-specific primers F1 and R1 used for PCR amplification. Filled boxes reflect exons of coding sequences, unfilled boxes are untranslated regions, and lines connecting exons represent introns.

(B) cDNA products amplified with F1 and R1 indicate reduced transcript size in las4 compared to the wild-type control (C).

(C) Sequencing of amplified cDNA products verified an in-frame 123 bp deletion (nucleotides 719-861) in VPS35B transcripts corresponding to the loss of entire exon 8. Alignment corresponds to nucleotides 400-1200 of wild-type VPS35B coding sequence. D, Sequence alignment of translated cDNAs reveals deletion of amino acids 247-287 from VPS35B protein in the las4 mutant.
Supplemental Figure 3. Analysis of VPS35B transcript levels in T-DNA mutant alleles vps35b-1 (SALK_014345) and vps35b-2 (GABI_784C05).

(A) Structure of the VPS35B gene showing positions of T-DNA insertions (open triangles) and primers (not to scale) used for real-time quantitative RT-PCR.

(B)-(C) VPS35B transcript levels in Col-WT, vps35b-1 and vps35b-2 using gene specific primer pairs positioned either (B) on exon 4 and 5 (F2, R2) or (C) in the 3’-UTR (F3, R3).
Supplemental Figure 4. Single loss-of-function alleles in VPS35 genes do not suppress PCD in acd11-2 in the absence of nahG.

Col-0 wild-type (WT), vps35a-1 (SALK_039689), vps35b-1 (SALK_014345), vps35b-2 (GABI_784C05), vps35c-1 (SALK_099735) (upper panel) as well as acd11-2, acd11-2 vps35a-1, acd11-2 vps35b-1, acd11-2 vps35b-2, acd11-2 vps35c-1 (lower panel) were grown for 33 days under short-day conditions at 21°C. Bar = 1 cm.
Supplemental Figure 5. Combined loss-of-function mutations in VPS35A and VPS35C do not suppress acd11 and lsd1 autoimmune cell death. 

(A) Col-0 wild-type (WT), vps35a-1 b-1, vps35b-1 c-1, vps35a-1 c-1 (upper panel) as well as acd11-2, acd11-2 vps35a-1 b-1, acd11-2 vps35b-1 c-1, and acd11-2 vps35a-1 c-1 (lower panel) were grown for 4 weeks under short-day conditions at 21°C.

(B) Col-0 WT, lsd1-2, vps35a-1 c-1 and lsd1-2 vps35a-1 c-1 plants were grown for 24 days under short day conditions and treated three times with 100 µM BTH. Photographs were taken 9 days after first treatment in comparison to untreated controls (upper panel). Scale bars indicate 1 cm.
Supplemental Figure 6. Phenotypes of retromer deficient mutants. Different vps35 double mutant combinations and mag1-1 plants were soil-grown for 60 days under short-day conditions. vps35a-1 c-1 plants show signs of early senescence which are absent from similarly dwarfed vps35a-1 b-1 and mag1-1 plants. Scale bars indicate 2 cm.
Supplemental Table 1. Oligonucleotides used in Supplemental Figures S1 and S3.

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SUPPLEMENTAL METHODS

Cloning and generation of transgenic plants

A 7.6 kb fragment of VPS35B was amplified from genomic DNA (Ler acd11 nahG) using PhusionTaq DNA Polymerase (Finnzymes), cloned into pENTR/D-TOPO (Invitrogen Life Technologies, Carlsbad, CA, US) and recombined via the LR reaction into the gateway destination vector pGWB501 (Nakamura et al., 2009). Cloning primers were 5’-TCTCATCATGTGTTCACTGTGATCAG-3’ and 5’-CTTAGACAAACGAAGAACATCTTGAGATAG-3’. Constructs were verified by sequencing, electroporated into Agrobacterium tumefaciens strain GV3101 and transformed into laz4-1 acd11-2 nahG by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MS-plates with 20 µg/mL hygromycin B, and transferred to soil.

Quantitative RT-PCR

Total RNA was extracted from frozen samples using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer’s protocol. Prior to cDNA synthesis, all RNA samples were DNase treated using the Turbo DNA-free Kit (Ambion,
Yield and integrity of the RNA were assessed using a NanoDrop micro photometer (NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis. First-strand cDNA was synthesized from 1 µg of total RNA by combined random hexamer and oligo dT priming using the First Strand cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA). Oligonucleotide primers of VPS35B and two reference genes PP2A (At1g13320) and RNA Helicase (At1g58050) (Czechowski et al., 2005) were designed and their amplification efficiency optimized (Supplemental Table S1). Quantitative PCR reactions were performed using iCycler MyiQ2 (Bio-Rad, Hercules, CA, USA) and DyNAamo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR amplifications were repeated at least twice on independent RNA samples. Calculations and statistical analyses were carried out as described in ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems, Austin, Texas, USA) (Avrova et al., 2003).

SUPPLEMENTAL REFERENCES


Title

Autophagy deficiency leads to accumulation of ubiquitinated proteins, ER stress and cell death in *Arabidopsis*

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Keywords

Autophagy, cell death, senescence, ubiquitin, infection, atg, age, npr1, ER stress
Abbreviations
ATG, autophagy related; ATG8-PE, ATG8-phosphatidylethanolamine; EDS1, enhanced disease susceptibility; ER, endoplasmic reticulum; HR, hypersensitive response; NPR1, non-expressor of PR genes; Pst, Pseudomonas syringae pv. Tomato; R, resistance; SA, salicylic acid; SAR, systemic acquired resistance; SID2, salicylic acid induction deficient; TM, tunicamycin; UPR, unfolded protein response; WT, wild type

Abstract
Autophagy is a homeostatic degradation and recycling process that is also involved in defense against microbial pathogens and in certain forms of cellular suicide. Autophagy has been proposed to negatively regulate plant immunity-associated cell death related to the hypersensitive response (HR), as older autophagy-deficient mutants are unable to contain this type of cell death 5-10 days after infection. Such propagating cell death was found to require non-expressor of PR genes (NPR1), but did not occur in younger atg mutants. In contrast, we find that npr1 mutants are not impaired in rapid programmed cell death activation upon pathogen recognition. We also provide molecular evidence that the NPR1 dependent spreading cell death in older atg mutants originates from an inability to cope with excessive accumulation of ubiquitinated proteins and ER stress which derive from salicylic acid (SA)-dependent systemic acquired resistance (SAR). Thus, autophagy functions in pro-survival but does not regulate cell death via a negative feedback loop through NPR1. Instead, npr1 loss-of-function indirectly rescues older atg mutants because atg npr1 double mutants are insensitive to SA and fail to
induce SAR that would otherwise produce terminal stress in autophagy deficient cells.

Introduction

Autophagy is a bulk degradation process involved in the recycling of nutrients, clearance of aggregates and responses to various stresses.\(^1\)\(^-\)\(^3\) Autophagic recycling at basal levels maintains physiological concentrations of nutrients via protein and lipid recycling, and autophagic activity may be augmented to remove dysfunctional organelles.\(^1\)\(^,\)\(^2\) Several autophagic variants have been reported, including macro-autophagy (hereafter termed autophagy), which is present in many organisms including animals and plants.\(^2\) This process relies on the concerted action of autophagy-related (ATG) genes to form double-membrane autophagosomes that fuse with vacuoles or lysosomes to degrade their internal cargo.\(^2\) Autophagosome assembly involves ATG8-phosphatidylethanolamine (ATG8-PE) conjugates, which are targeted to autophagosomal membranes, and levels of lipidated ATG8 can be proportional to the number of autophagosomes formed.\(^2\) In *Arabidopsis thaliana*, the protein NBR1 binds to autophagosomal bound ATG8 and acts as a cargo receptor that targets ubiquitinated protein aggregates for autophagic degradation. NBR1 itself is also a selective substrate of autophagy.\(^4\)\(^,\)\(^5\)

The early senescence phenotype of loss-of-function *atg* mutants indicate that autophagy is required to maintain cellular homeostasis in response to internal and external cues (Fig. S1).\(^6\)\(^,\)\(^7\) However, the concurrent functions of autophagy complicate analyses of its contributions to responses to different stimuli. Nonetheless, it has been established in metazoans that autophagy, together with the endoplasmic reticulum (ER) stress response, is one of the earliest examples of
innate immune responses to combat intruders. In addition, autophagy is implicated as a mechanism for cellular suicide in several organisms.

Plants rely on a multilayered defense system in which cytoplasmic receptors termed resistance (R) proteins recognize pathogen effectors or their virulence activities to trigger host immune responses, including rapid and restricted cell death at the site of pathogen entry. This form of pathogen-induced cell death is known as the HR. Several reports have documented a gradual spread of cell death in atg mutants from infected to uninfected tissues which develops over several days following pathogen inoculation. However, it has also been shown that rapid development of HR cell death is suppressed in infected tissues of autophagy-deficient mutants, and that autophagy can promote HR cell death. More specifically, both Hofius et al. and Yoshimoto et al. noted that cell death triggered by the resistance protein RPM1 does not spread beyond infection sites in younger atg mutants (4-5 weeks, before the onset of early senescence), while propagating cell death occurs several days after infection in older atg mutants (7-8 weeks, when early senescence is well established).

Yoshimoto et al. demonstrated that levels of the phytohormone SA increased along with transcripts of senescence-associated SAG12 and of the SA responsive markers PR1 and PR2 in atg5 and atg2 mutants. These increases correlated with the onset of a visible early senescence phenotype compared to wild type (WT) plants. Senescence-associated cell death and growth retardation of atg mutants could be reversed by expression of the bacterial SA hydroxylase nahG, as well as by mutations in the SA biosynthetic gene salicylic acid induction deficient2 (SID2), or in the SA receptor component NPR1 (Fig. S1). Importantly, suppression of the propagation
and apparently immunity-related cell death in atg mutants was also observed in SA- and NPR1-deficient backgrounds.\(^6,18\) In addition, Yoshimoto et al.\(^6\) observed that application of the SA agonist BTH restored the accelerated cell death phenotype in atg5 nahG and in atg5 sid2 double mutants, but not in atg5 npr1. This led them to conclude that autophagy regulates a novel, negative feedback loop through NPR1, which modulates SA signaling and thereby limits senescence and immunity-associated cell death. This conclusion is surprising because npr1 mutants have been shown to exhibit much stronger immunity-associated cell death than WT plants upon avirulent infection.\(^19\)

Results

Since propagating cell death in older atg mutants is first macroscopically visible several days post infection, and as NPR1 has previously been implicated in restricting HR cell death triggered by RPM1,\(^19,20\) we investigated the importance of NPR1-dependent HR cell death at primary infection sites. We monitored cell death development in infected leaves of npr1 single and atg5 npr1 double mutants. Cell death induced by Pseudomonas syringae pv. Tomato (Pst) DC3000 carrying AvrRpm1 was quantified using an electrolyte leakage assay.\(^21\) As previously reported,\(^19\) hypersensitive cell death in npr1 exceeded the levels observed in WT controls (Fig. 1A). This confirms that NPR1 is not required for RPM1 triggered HR cell death. In contrast, HR cell death in atg5 mutants was slightly but significantly suppressed compared to WT, as previously observed.\(^12\) Surprisingly, as for npr1, HR cell death in the atg5 npr1 double mutant was also significantly higher than WT. This again indicates that NPR1 is not required for the promotion of
HR cell death. To verify the effects of autophagy deficiency, we extended our analysis by measuring HR cell death in \textit{atg2} and in \textit{atg2 npr1} double mutants. \textit{atg2} mutants exhibit a more severe phenotype than \textit{atg5},\textsuperscript{6,18} which is in agreement with recent reports of residual autophagy activity in \textit{atg5} and \textit{atg7} mutants.\textsuperscript{22} As seen in Figure 1B, RPM1 triggered HR cell death was strongly suppressed in \textit{atg2} mutants compared to WT, but \textit{npr1} single mutants exhibited significantly more ion leakage than both WT and the single \textit{atg2} mutant. In contrast, the \textit{atg2 npr1} double mutant displayed an intermediary level of ion leakage. Since \textit{atg2} mutants are autophagy-deficient, NPR1 must be needed to dampen autophagy-independent types of HR cell death because cell death in \textit{atg5 npr1} and \textit{atg2 npr1} double mutants exceeds cell death in \textit{atg5} and \textit{atg2} single mutants (Fig. 1A + 1B). We note that primary HR cell death is also similarly suppressed in older \textit{atg} mutants, and that we observe no marked differences between genotypes if ion leakage measurements are extended to 12 hours (data not shown). Nevertheless, these observations are also supported by microscopic analysis of HR cell death stained with trypan blue 12 hours after infection with low doses of \textit{Pst DC3000 (AvrRpm1)}\textsuperscript{23} \textit{Atg2} mutants exhibit the lowest visible level of HR cell death and \textit{atg2 npr1} exhibits WT-like levels of cell death, while most leakage is seen in \textit{npr1} single mutants (Fig. 1C). This is in agreement with the results of Rate and Greenberg,\textsuperscript{19} and is also supported by their finding that plants which overproduce NPR1 exhibit a weaker HR. Most importantly, HR cell death triggered by RPM1 is both temporally and genetically discrete from the type of cell death seen in adjacent, uninfected tissues of older \textit{atg} mutants. To exclude the possibility that major differences in bacterial growth may partially explain our observations, we assayed bacterial growth 6 hours post infection. This
revealed negligible bacterial growth during the first 6 hours, and no significant difference in that time frame for all genotypes tested, apart from the *rpm1* knockout control compared to WT (Fig. S2). These results raise at least two further questions: 1) why is the early senescence phenotype of *atg* mutants suppressed by mutation of NPR1 (Fig. S1), and 2) why do cells in uninfected tissue of older *atg* mutants die some days after BTH treatment or when RPM1, and probably also other resistance proteins, are triggered.

First, since *atg* mutants lack a basic metabolic pathway for recycling accumulating cellular ‘debris’, older *atg* mutant leaves accumulate metabolites, oxidized proteins and lipid peroxides, and thus experience increasing oxidative stress. Lipid peroxides, for example, are potent inducers of *PR* gene, and intracellular oxidative stress can trigger an inappropriate rise in SA followed by defence activation and disease responses. Such cellular stress would likely be self-reinforcing in autophagy-deficient backgrounds, and autophagy-deficient mutants would consequently senesce earlier. Since NPR1 is required for SA induced defence responses, such a positive feedback loop should be suppressed by mutation of NPR1. If so, then the accumulation of non-recycled proteins should be higher in *atg2* single than in *atg2 npr1* double mutants.

To test this possibility, we assayed the accumulation of the autophagosomal membrane marker ATG8. Both lipidated and non-lipidated ATG8 accumulate in *atg*-deficient mutants for two reasons: *i.* autophagy is induced by the build-up of cellular ‘debris’, and *ii.* forms of ATG8 persist because autophagy is incomplete. In line with this, marked accumulation of ATG8 was detected in three week old *atg2* and *atg2 npr1* mutants, but only trace amounts of ATG8 accumulated in *npr1* (Fig. 2A). However, at 6 weeks and particularly at 9 weeks, ATG8 was
more abundant in \textit{atg2} than in \textit{atg2 npr1} mutants (Fig. 2A).

We then assayed whether there was differential accumulation of the autophagy cargo adaptor NBR1, which contains a UBA domain capable of binding ubiquitinated substrates to target them for degradation through autophagy.\textsuperscript{4,5} Marked accumulation of endogenous NBR1 could be detected by immuno-blotting with an anti-NBR1 antibody in 3 week old \textit{atg2} and \textit{atg2 npr1} mutants but not in \textit{npr1} (Fig. 2B). In 6 week old plants, slightly more NBR1 was observed in \textit{atg2} compared to \textit{atg2 npr1}. This trend increased markedly at 9 weeks when NBR1 forms recognised by the antibody were much more abundant in \textit{atg2} than \textit{atg2 npr1} double mutants (Fig. 2B). As in \textit{npr1}, NBR1 did not accumulate in WT at any of the time points tested (See untreated samples in Fig. 3B).

In addition, we assayed whether increased levels of NBR1 correlated with the levels of ubiquitinated proteins normally degraded via autophagy. Marked differences in the levels of ubiquitinated proteins were not detectable in 3 week old \textit{npr1}, \textit{atg2} and \textit{atg2 npr1} mutants (Fig. 3A; Fig. S3A). Interestingly, in older plants there was a gradual accumulation of ubiquinated proteins in \textit{atg2} that was more pronounced than in WT and \textit{npr1} (Fig. 3A). More importantly, this accumulation was greater in \textit{atg2} than in \textit{atg2 npr1}, and the difference was more pronounced in 9 week old than in 6 week old plants (Fig. 3A; Fig. S3B). Thus, a steady build-up of ubiquitinated proteins in autophagy-deficient backgrounds can be alleviated by introducing loss-of-function mutations such as \textit{npr1} in the SA pathway. These observations were confirmed by probing the blots using an alternative anti-ubiquitin antibody (Fig. S3B).

Plants respond to \textit{Pst DC3000 (AvrRpm1)} and other avirulent pathogens by inducing SAR
which leads to increased SA and defense gene transcript levels in uninfected tissues.\textsuperscript{28} NPR1, a central component of SA reception, controls the expression of \textit{PR} genes and a large set of SAR responsive, ER resident proteins. These ER genes are upregulated to ensure proper folding and secretion of the high levels of PR proteins required to establish SAR.\textsuperscript{27,29} Autophagy is needed to counterbalance ER stress and to remove misfolded proteins.\textsuperscript{30} It is therefore probable that SAR induced by infection, or by treatment with the SA analog BTH, leads to deleterious hyper-accumulation of unprocessed PR proteins, and thus of NBR1 selective cargo and ubiquitinated proteins, in \textit{atg} mutants. To test this, we compared the accumulation of NBR1 and ubiquitinated proteins in 6 week old plants before and after BTH treatment. Similar to our earlier observations, NBR1 and ubiquitinated proteins were more abundant in untreated \textit{atg2} than in \textit{atg2 npr1, npr1} or WT (Fig. 3B + 3C). However, a dramatic accumulation of NBR1 and ubiquitinated proteins was observed in \textit{atg2} plants 24 hours after treatment with BTH. More importantly, such BTH-induced accumulation was almost completely absent in \textit{atg2 npr1} (Fig. 3B + 3C). This demonstrates that NPR1 loss-of-function is also sufficient to abrogate accumulation of ubiquitinated proteins in autophagy-deficient mutants and to thus hinder disruption of cellular homeostasis.

SA treatment was previously shown to be sufficient to cause tissue collapse and cell death in \textit{bip2} mutants defective in ER protein folding and secretory capacity.\textsuperscript{29,31} Importantly, the SA-induced ER stress and leaf collapse phenotypes of \textit{bip2} mutants was caused by insufficient processing of PR proteins, and could thus be rescued by \textit{npr1}. This rescue is very similarly to what we have described above for \textit{atg} mutants which have been shown in other model systems to
be hypersensitive to ER stress.\textsuperscript{32} This may explain why NPR1 mutation suppresses both the spread of lesions triggered by immune responses, as well as senescence-associated cell death in \textit{atg} mutants. Increased expression of proteins destined for secretion during the immune response triggers the unfolded protein response (UPR) in the ER.\textsuperscript{29} One induced component of this response is bZIP60, a transcription factor which regulates the expression of proteins involved in protein folding and degradation. bZIP60 is synthesized from mRNA spliced by IRE1, and bZIP60 mRNA splicing is stimulated by ER stress inducers.\textsuperscript{33-35} Thus, in addition to a regular, unspliced bZIP60 amplicon (bZIP60u), a smaller spliced amplicon (bZIP60s) can be seen in RT-PCR assays performed on plants treated with ER stress inducers. To test if ER stress is lower in NPR1-deficient backgrounds, we assessed the expression of these two forms of bZIP60 mRNA around the time when levels of ubiquitinated proteins become apparent (Fig. 3C). As seen in Fig. 4, we observed no major differences among the various genotypes in the expression of unspliced bZIP60 transcripts in both 6 and 7 week old plants using RT-PCR. In contrast, the levels of spliced bZIP60 were lower in \textit{npr1} and \textit{atg2 npr1} mutants than in WT, and markedly lower than \textit{atg2} mutants. Interestingly, the difference between WT and \textit{atg2} was lower in 7 week old than in 6 week old plants. This indicates that, as they age, ER stress in WT plants rises to a level more close to that of an autophagy-deficient background (Fig. 4). However, the comparably low levels of spliced bZIP60 in \textit{npr1} and \textit{atg2 npr1} indicate that the ER experiences a smaller flux of proteins in NPR1-deficient backgrounds. It also provides additional evidence for how NPR1 loss-of-function rescues the spread of cell death in uninfected SAR tissue, as defense gene products are not upregulated in \textit{npr1}, thus minimizing the pressure on the ER.\textsuperscript{29}
Autophagy also functions as a pathway for the turnover of ER membranes and contents in response to ER stress in *Arabidopsis*. Thus, treatment with tunicamycin (TM), a potent inducer of ER-stress that inhibits protein N-glycosylation, leads to the accumulation of autophagosomes. Our findings indicate that compromised homeostasis in phenotypically pre-senescent and in ER stressed *atg* mutants primes them to succumb to additional stress or injury. If so, then it should be possible to combine ER stress with an additional stress such as BTH treatment to produce lethal effects in younger *atg* mutants. To examine this, we germinated WT, *atg2*, *npr1* and *atg2 npr1* seedlings on MS plates containing TM and BTH. This revealed that young *atg2* single mutants were unable to cope with these combined stresses and began to die within 4 weeks (Fig. 5A). In contrast, loss of NPR1 function rescued these effects such that *atg2 npr1* double mutants survived. Similar observations were made with *atg5* and *atg5 npr1* (Fig. S4). Since *atg2* seeds germinated to a lower extent than other genotypes on plates with both TM and BTH, it was possible that individual *atg2* seedlings were exposed to higher TM and BTH concentrations in their immediate surroundings compared to the other genotypes (Fig. 5A). To clarify this, we seeded all genotypes on MS plates and subsequently transferred the germinated seedlings individually to MS plates containing TM and BTH. This demonstrates that *atg2* mutants succumb to the same concentrations of TM and BTH tolerated by the other genotypes (Fig. S5).

Finally, we also assessed if it was possible to mimic the uncontrolled, age-dependent spread of cell death observed upon infection with avirulent pathogens. We therefore injected TM into one side of leaves of 4 week old WT, *npr1*, *atg2 npr1* and *atg2* plants, waited 5 days for
the genotypes to cope with this stress, and then injected avirulent Pst (AvrRpm1) in the other side of the leaves. Surprisingly, while we observed contained HR-associated lesions develop in WT, npr1 and atg2 npr1 genotypes after 4 days, the atg2 genotype was unable to contain such cell death (Fig. 5B). This spread of cell was not observed in young atg2 plants if Pst (AvrRpm1) or TM was injected alone. Thus, when atg mutants reach high levels of ER stress, subsequent additive stressors, such as infection, will be detrimental and result in cell death and lesions (Fig. 5B).

Discussion

In agreement with previous reports,12,37 we also observe less cell death in atg mutants. Interestingly, this is suppressed by mutations in npr1 and, since atg2 npr1 mutants are also autophagy deficient (Figure 3A), NPR1 must suppress autophagy independent cell death mechanisms. Mutations in another important mediator of the SA signaling pathway, enhanced disease susceptibility1 (EDS1), likewise accelerate HR cell death triggered by RPM1.12 While the molecular basis for these observations remains unclear, it is evident that HR cell death may be executed via more than one signaling pathway.12,38

Most importantly, we provide evidence that cell death outside of infected areas in older atg mutants is probably caused by SAR in uninfected cells with compromised homeostasis. Thus, autophagy does not appear to regulate a negative feedback loop through NPR1 as previously proposed.6 Were the latter true, then it would also imply that autophagy regulates cell death through NPR1 in one manner in the HR, a different manner in adjacent tissues, and differently
again in younger plants compared to older plants. We also conclude that autophagy-deficient mutants die before WT plants because of an inappropriate accumulation of ubiquitinated protein aggregates and increasing ER stress. This deficiency puts them at risk,\textsuperscript{5,6,39} and disruption of cellular homeostasis leads to SA buildup and NPR1-dependent accumulation of defense related transcripts\textsuperscript{6} in a deleterious cycle that is suppressed by mutations in NPR1. Thus, it appears to be problematic to draw conclusions about autophagic function of developmentally older $atg$ mutants, due to secondary effects of their long-term, compromised cellular homeostasis. Analyses of inducible knockout mutants may therefore be a better way to address questions of how autophagy regulates cellular processes in adult plants.
Materials and Methods

Plant Material and Growth Conditions

Plants grown on soil were kept in environmental chambers under 8 hours of light (150 mE/m²/s) at 21°C and 70% relative humidity, after seed surface sterilization with 70% ethanol. Seedlings grown on solid MS medium (0.44% w/v agar, 1% w/v sucrose, pH5.7) were kept under 16 hours of light (150 mE/m²/s) at 21°C, after seed surface sterilization with 1.3% v/v bleach followed by 70% ethanol. Seeds germinated on MS plates were supplemented with 0.00005% (v/v) DMSO (Mock), 0.005 µg/mL tunicamycin, 50 µM BTH or 5 ng/mL tunicamycin and 50 µM BTH. *Arabidopsis* knockout mutants were *rpm1*, *npr1*, *atg2* and *atg2 npr1* and *atg5* and *atg5 npr1*. All data result from at least three independent experiments.

Bacterial assays

Ion leakage assays and trypan blue staining following syringe-infiltration of the avirulent *Pst* DC3000 (*AvrRpm1*) strain were performed with 1 x 10⁸ or 1 x 10⁶ CFU mL⁻¹ as previously described. Bacterial growth assay following syringe-infiltration with avirulent *Pst* (*AvrRpm1*) were performed at 1 x 10⁸ CFU mL⁻¹, as previously described.

Lesion development

One side of leaves from 4 week old plants were infiltrated with 100 ng/mL tunicamycin,
followed by an injection in the opposite side of the leaf 5 days later, with avirulent *Pst (AvrRpm1)* at $2 \times 10^7$ CFU mL$^{-1}$. Representative pictures were taken 4 days after the last injection.

Immunoblotting

Total proteins were extracted in urea buffer (4M urea, 100mM DTT, 1% Triton X-100) using a mortar and pestle. Samples were spun 10 minutes at 15,000g and 3x SDS loading buffer (30% glycerol, 3% SDS, 94 mM Tris pH 6.8, 75mM DTT) was added to the supernatant. For anti-Ubiquitin immunoblots, total protein extracts were separated by 10% SDS-PAGE, blocked with 5% high grade BSA or 2% gelatine for 1 hour and subsequently probed with anti-Ubiquitin antibody (Fig. 3, Dako, Z0458; Fig. S3, Agrisea, AS08 307; 0.02% sodium azide) followed by anti-Rabbit-HRP conjugated antibody (Promega, W4028). For anti-NBR1 immunoblots, total protein extracts were separated in 10% SDS or 12% 6M urea gels by SDS-PAGE, blocked with 5% milk or 5% high grade BSA for 1 hour, and subsequently probed with anti-NBR1 antibody (kindly provided by T. Johansen, Tromsø, Norway; 1% BSA, 0.02% sodium azide) followed by anti-Rabbit-HRP conjugated antibody (Promega, W4018). For anti-ATG8a immunoblots, total protein extracts were separated on 6M 12% urea gels using SDS-PAGE, blocked with 5% milk for 1 hour, and subsequently probed with anti-ATG8a antiserum (kindly provided by Y. Ohsumi, Okazaki, Japan; 1% BSA, 0.02% sodium azide) followed by anti-Rabbit-AP conjugated antibody (Promega, S3738; 1% milk). Amido black or coomassie blue staining of the large subunit of RuBisCO serve as loading control.
RT-PCR

Total RNA was extracted using Omegas Plant RNA kit and cDNA synthesized using Life SuperScript III Reverse Transcriptase kit. Unspliced and spliced bZIP60 transcript levels were analyzed by RT-PCR as previously described.\textsuperscript{34,35}

Statistics

Statistical analyses were performed with an ANOVA with post hoc Holm-Šidák test, using GraphPad Prism 6. Significance was accepted at the level of \( p \leq 0.05 \).

ACKNOWLEDGMENTS

We thank Dingzhong Tang and Kokhi Yoshimoto for the \textit{atg2} and \textit{atg2 npr1} knockout seeds, and Kokhi Yoshimoto for the \textit{atg5} and \textit{atg5 npr1} knockout seed lines. We thank Terje Johansen for the NBR1 antibody, and Yoshinori Ohsumi for the ATG8a antiserum. We also thank Suksawad Vongvisuttikun for technical assistance. This work was funded by grants to MP from the Danish Council for Independent Research (11-106302) and to ER by the Portuguese Foundation for Science and Technology (FCT) (SFRH/BPD/75696/2011).
REFERENCES


Figure legends

Figure 1. A + B) Ion leakage assays of (A) 6 week old Col-0 WT, atg5, npr1 and atg5 npr1 plants and (B) 6 week old Col-0 WT, rpm1, atg2, npr1 and atg2 npr1 plants, after inoculation with Pst DC3000 (AvrRpm1). Mean and standard errors (SE) were calculated from four or six discs per treatment with 3 or 4 replicates within an experiment. Experiments were repeated with similar results. Pairwise comparisons at the last time point post infection for means versus WT were performed with one-way ANOVA test followed by Holm-Šidák post hoc test. a, P ≤ 0.05, b, P ≤ 0.01, c, P ≤ 0.0001. C) Leaves from Col-0 WT, npr1, atg2, atg2 npr1 and rpm1 stained with lactophenol-trypan blue, 12 hours after infection with Pst DC3000 (AvrRpm1). Size bar represents 2.5mm.

Figure 2. Immunoblot detection of (A) ATG8a and (B) NBR1 accumulation in npr1, atg2 npr1 and atg2 plants, 3, 6 and 9 weeks old. Coomassie blue staining of the large subunit of RuBisCO serve as loading control.

Figure 3. A) Immunoblot detection of ubiquitin in 3, 6 and 9 week old WT, npr1, atg2 npr1 and atg2 plants. B + C) Immunoblot detection of (B) NBR1 and (C) ubiquitin in 7 week old WT, npr1, atg2 npr1 and atg2 plant, after 24 hours of treatment with mock or 100 µM BTH. Amido black or coomassie staining of the large subunit of RuBisCO serve as loading control. Anti-ubiquitin antibody from Dako.
Figure 4. RT-PCR of unspliced and spliced bZIP60 mRNA levels in Col-0 WT, npr1, atg2 and atg2 npr1, in 5 and 7 week old plants. Actin serves as loading control.

Figure 5. A) 4 week old Col-0 WT, npr1, atg2 and atg2 npr1 plants grown on MS plates supplemented with 0.00005% DMSO (Mock), 5 ng/mL tunicamycin, 50 µM BTH or 5 ng/mL tunicamycin and 50 µM BTH. B) 4 week old WT, npr1, atg2 npr1 and atg2 leaves injected in one side with 100 ng/mL tunicamycin (“Tunicamycin” and “AvrRpm1/Tunicamycin”) or 0.00005% DMSO (“AvrRpm1”), and then 5 days later injected with Pst (AvrRpm1) at 2 x 10^7 CFU mL⁻¹ (“AvrRpm1” and “AvrRpm1/Tunicamycin”) or 10 mM MgCl₂ (“Tunicamycin”), in the opposite side of the leaves. Pictures taken 4 days after the Pst (AvrRpm1) or MgCl₂ injection.

Fig. S1. Col-0 WT, npr1, atg2 and atg2 npr1 plants 5, 7 and 9 weeks old.

Fig. S2. Growth of avirulent Pst DC3000 (AvrRpm1) in 5 week old WT, npr1, atg2, atg2 npr1 and rpm1 plants. Plants were inoculated with 1 x 10⁸ colony forming units (CFU) mL⁻¹ and log10 bacterial counts per area of leaf plotted against 0 and 6 hours post infection (HPI). Error bars indicate standard deviation calculated from the mean of 3 samples per genotype. Pairwise comparisons for all means at the 6 hour time point post infection were performed with a one-way ANOVA test followed by Tukey post hoc test. n.s., not significant. a, P ≤ 0.05.

Fig. S3. A) Increased exposure of the HRP-developed immunoblot of ubiquitin from 3 week old
tissue from WT, npr1, atg2 npr1 and atg2 plants, shown in Figure 3A. Amido black staining of the large subunit of RuBisCO serves as loading control. Anti-ubiquitin antibody from Dako. B) Two different exposures of the same HRP-developed immunoblot of ubiquitin from 6 week old WT, npr1, atg2 npr1 and atg2 plants. Amido black staining of the large subunit of RuBisCO serve as loading control. Anti-ubiquitin antibody from Agrisera.

Fig. S4. 5 week old Col-0 WT, npr1, atg2 and atg2 npr1 plants grown on MS plates supplemented with 5 ng/mL tunicamycin and 50 µM BTH.

Fig. S5. Single seedlings of Col-0 WT, npr1, atg2 npr1 and atg2 grown 12 days on MS plates, followed by the transfer of single seedlings to MS plates supplemented with 5 ng/mL tunicamycin and 50 µM BTH, and subsequently grown for an additional 16 days before pictures were taken.
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# Cell Reports

**Arabidopsis accelerated-cell-death11, ACD11, is a ceramide-1-phosphate transfer protein and intermediary regulator of phytoceramide levels**  
--Manuscript Draft--

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Rhoderick E Brown |
| Abstract:          | The accelerated-cell-death11 (acd11) mutant of Arabidopsis provides a genetic model for studying immune response activation and localized cellular suicide that halts pathogen spread during infection in plants. Here, we elucidate ACD11 structure/function and show that acd11 disruption dramatically alters the in vivo balance of sphingolipid mediators that regulate eukaryotic programmed cell death. In acd11 mutants, normally low ceramide-1-phosphate (C1P) levels become elevated, but the relatively abundant cell death inducer, phytoceramide, rises acutely. ACD11 exhibits selective intermembrane transfer of C1P and phyto-C1P. Crystal structures establish C1P binding via a surface-localized, phosphate head-group recognition center connected to an interior hydrophobic pocket that adaptively ensheaths lipid chains via a cleft-like gating mechanism. Point mutation mapping confirms functional involvement of binding-site residues. A π helix (π-bulge) near the lipid-binding cleft distinguishes apo-ACD11 from other GLTP-folds. The global two-layer, α helically-dominated, 'sandwich' topology displaying C1P-selective binding identifies ACD11 as the plant prototype of a new GLTP-fold subfamily. |
December 9, 2013

Korie Handwerger, Ph.D.
Scientific Editor, Cell Reports

Re: CELL-REPORTS-D-13-00660

"Arabidopsis accelerated-cell-death11, ACD11, is a ceramide-1-phosphate transfer protein and intermediary regulator of phytoceramide levels"

Dear Dr. Handwerger,

We are very pleased to learn of that our manuscript (Cell-Reports-D13-00660) has been "accepted in principle" pending receipt and approval of the stipulated specific file set that conforms to Cell Reports guidelines. We thank you for explaining the unusual situation regarding the review process that led to the atypical delays.

As requested, we have revised our manuscript to include the Protein Data Bank (PDB) accession numbers for the five reported protein crystal structures (pg. 15) and to meet other set-up requirements stipulated in the guideline links in your cover letter. We have been especially attentive to the guidelines checklist. Changes include the omission of parentheses from title lines and a comprehensive effort to reduce the manuscript length to achieve the desired 55,000 characters + spaces. Please note that, prior to the recent reductions, the revised manuscript count had expanded to 61,059 because of the new discussions and experiments requested by Referees. The current count remains a bit long for the main text (56,914 w/o title page + Highlights) for the Cell Reports format. We are hopeful that a bit of leeway can be granted, given our strong efforts to comply, i.e. reduction by ~4,145. Since no new scientific issues were raised during review of our revised manuscript, the scientific content remains unaltered.

We are grateful for the helpful comments provided by the Reviewers and for your efforts during the review process. We hope that our manuscript now can be officially accepted for publication in Cell Reports.

Sincerely,

Rhoderick E. Brown  Dinshaw J. Patel  John Mundy
Univ. Minnesota  MSKCC  Univ. Copenhagen
Our manuscript has been revised to include the Protein Data Bank (PDB) accession numbers for the five reported protein crystal structures (pg. 15)

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Arabidopsis ACD11 is a Ceramide-1-phosphate transfer protein

Arabidopsis thaliana (4 weeks)

control

acd11 null mutant

The π-helix-to-α-helix transition that occurs in ACD11 during ceramide-1-phosphate binding is unique among GLTP-folds
HIGHLIGHTS

- The *acd11* mutant of *Arabidopsis* provides a genetic model for cell suicide in plants
- ACD11 forms a GLTP-fold and functions as a ceramide-1-phosphate transfer protein
- Apo-ACD11 is a unique GLTP-fold due to a π-bulge located near the lipid binding site
- In *acd11* mutants, ceramide-1-phosphate increases but phytoceramide rises acutely
Arabidopsis accelerated-cell-death11, ACD11, is a ceramide-1-phosphate transfer protein and intermediary regulator of phytoceramide levels

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SUMMARY
The accelerated-cell-death11 (acd11) mutant of Arabidopsis provides a genetic model for studying immune response activation and localized cellular suicide that halts pathogen spread during infection in plants. Here, we elucidate ACD11 structure/function and show that acd11 disruption dramatically alters the in vivo balance of sphingolipid mediators that regulate eukaryotic programmed cell death. In acd11 mutants, normally low ceramide-1-phosphate (C1P) levels become elevated, but the relatively abundant cell death inducer, phytoceramide, rises acutely. ACD11 exhibits selective intermembrane transfer of C1P and phyto-C1P. Crystal structures establish C1P binding via a surface-localized, phosphate headgroup recognition center connected to an interior hydrophobic pocket that adaptively ensheaths lipid chains via a cleft-like gating mechanism. Point mutation mapping confirms functional involvement of binding-site residues. A π-helix (π-bulge) near the lipid-binding cleft distinguishes apo-ACD11 from other GLTP-folds. The global two-layer, α-helically-dominated, ‘sandwich’ topology displaying C1P-selective binding identifies ACD11 as the plant prototype of a new GLTP-fold subfamily.

RUNNING TITLE
Arabidopsis ACD11 Structure and Function
INTRODUCTION

Sphingolipids and their metabolites, i.e. ceramide (Cer), ceramide-1-phosphate (C1P), and the long chain bases (LCB), sphingosine and sphingosine-1-phosphate (S1P), are bioactive lipids that function as messenger signals and mediators of eukaryotic processes such as cell growth, development, embryogenesis, senescence, inflammation, and programmed cell death (PCD) (Fyrst and Saba, 2010; Hannun and Obeid, 2008; Michaelson, 2010). The dynamic balance between Cer (sphingoid base amide-linked to a fatty acyl chain) and its phosphorylated derivative, C1P, critically regulates PCD in plants and animals (Berkey et al., 2012; Chen, 2009; Pata et al., 2010; Reape and McCabe, 2008).

In plants, PCD occurs during development, during disease symptoms associated with virulent infections, and during the hypersensitive response (HR) induced by avirulent stress effectors (Lam, 2004). Hallmarks of HR are local accumulation of reactive oxygen species, nitric oxide, and the phytohormone, salicylic acid. By inducing localized cell death triggered when resistance proteins recognize specific pathogen-derived molecules, HR potentiates defensive resistance. Mutants exhibiting accelerated cell death (acd) phenotypes in the absence of pathogen effectors also provide insights into HR-like PCD and defense activation. One HR mimic is the acd5 mutant, which lacks Cer kinase activity and accumulates Cers, triggering PCD (Liang et al., 2003). C1P addition partially abrogates the PCD-inducing effects of elevated Cer in acd5. In acd11 null mutant, HR-related PCD and defense genes are constitutively activated in salicylic acid-dependent fashion. The acd11 gene encodes ACD11, a lipid transfer protein able to moderately accelerate the intermembrane transfer of sphingosine and sphingomyelin, but not Cer or glycosylceramides (Brodersen et al., 2002; Petersen et al., 2008).

Structural homology modeling predicts ACD11 forms a GLTP-fold and is a glycolipid transfer protein (GLTP) superfamily member (Airenne et al., 2006; Brown and Mattjus, 2007; Petersen et al., 2008). Yet, ACD11 is unable to transfer glycolipids (Brodersen et al., 2002), consistent with the lack of essential residues needed for glycosphingolipid (GSL) sugar headgroup binding (Petersen et al., 2008). In mammalian GLTPs and HET-C2 fungal GLTP, X-ray structures reveal the molecular details of how glycolipids are recognized and bound by a conserved residue cluster (Asp, Asn, Lys, His, Trp) that form a hydrogen bond network with the GSL sugar-amide region, thus explaining the selectivity and transfer proficiency for various GSLs (Airenne et al., 2006; Kenoth et al., 2011; Kenoth et al., 2010; Malinina et al., 2006; Malinina et al., 2004; Samygina et al., 2011). Currently lacking for ACD11 is establishment of its preferred sphingolipid ligand as well as direct evidence for its functional involvement in the regulation of plant sphingolipid metabolism.
Herein, we investigated ACD11 structure and lipid transfer specificity and discovered high selectivity for C1P and phyto-C1P, but not related plant sphingolipids, i.e. glucosylceramides (GlcCer), Cer, glycosylinositolphosphoceramides (GIPC), and sphingoid long chain bases (LCB). X-ray structures establish ACD11 global architecture to be a GLTP-fold and reveal the molecular basis for selective recognition of C1P. Point mutation functional analyses support structural mapping showing a cationic residue cluster mediating the selective binding of the C1P phosphate headgroup in a surface-located recognition cavity. An intra-helical distortion, i.e. \( \pi \)-helix (\( \pi \)-bulge) uniquely distinguishes ACD11 from other known GLTP-folds including the recently discovered human ceramide-1-phosphate transfer protein (CPTP) (Simanshu et al., 2013). The \( \pi \)-bulge involves key residues of the C1P recognition center that regulates access and encapsulation of the lipid hydrocarbon chains to an adjoining hydrophobic pocket. In Arabidopsis acd11 null mutant, normally low C1P levels are elevated, while relatively abundant phytoceramides (phyto-Cer) rise acutely, consistent with shifts in the dynamic balance and distributions of these two sphingolipids playing a key role in plant PCD regulation.

RESULTS

ACD11 Forms a GLTP-fold with a Helical \( \pi \)-bulge in its Lipid Headgroup Recognition Center

To experimentally establish if ACD11 forms a GLTP-fold, we crystallized wild-type (wt) protein and determined its structure (1.8 Å) (Tables S1 and S2). ACD11 adopts the two-layer, all \( \alpha \)-helical ‘sandwich’ motif characteristic of the GLTP-fold (Figure 1A). Nonetheless, there are differences compared to the human GLTP-fold prototype (Figure S1). At the N-terminus, ACD11 has an extra \( \alpha \)-helix (designated \( \alpha \)N) that is lacking in human GLTP and is ~35% shorter in human CPTP. Key residues involved in lipid headgroup recognition in ACD11 (cyan) differ in GLTP (Figure 1B; pink) but not in CPTP (Figure 1C; beige) except for conserved Asp60 and His143, residues needed for Cer interaction in all GLTP-folds (Figure S1A; red). The ACD11 C-terminal region does not directly contribute to formation of the headgroup recognition cavity as occurs in GLTP (Figure 1B, red arrow) but resembles the HET-C2 fungal GLTP-fold, which terminates similarly with a Trp residue (Kenoth et al., 2010). In ACD11, C-terminal Trp206 positioning is stabilized by cation-pi interaction with Arg92, but no similar interaction occurs in GLTP or CPTP, which end with Val209 and Pro214, respectively. A relatively small, compact cavity for lipid headgroup binding exists in ACD11 and CPTP, a consequence of the \( \alpha \)3-4 loop projecting out and over in hood-like fashion (Figure 1B, blue arrow; 1C). The nearby surface region is highly basic (Figures 1D and 1F) compared to its more neutral counterpart in human...
GLTP (Figure 1E). A noteworthy and novel structural feature of apo-ACD11 is the π-helix (π-bulge) in helix α2 near Asp60 resulting in close proximity to His143 via a 2.9 Å salt bridge (Figures 1G and 1J). In all other known apo-GLTP-folds including CPTP, no π-bulge occurs and the analogous Asp and His residues remain further apart (Figure 1H and 1I).

Crystal Structure of ACD11 in Complex with Lysosphingomyelin

The first tests of ACD11 transfer of glycosphingolipid and related metabolites (Brodersen et al., 2002) preceded crystal structure determination of the human GLTP-fold and mapping of the glycolipid binding site (Malinina et al., 2004). GLTP and ACD11 superpositioning (Figure 1B) reveals a positively-charged residue triad (K64, R99, R103) in ACD11 replacing N52, L92, and W96 in GLTP. This explains the lack of glycolipid transfer by ACD11 and limited transfer of SM, which has a phosphocholine headgroup (Petersen et al., 2008). Thus, initial trials focused on co-crystallization of wtACD11 complexed with SM and lysoSM (Figure 2A). Only the latter lipid yielded a crystal complex enabling 2.4 Å resolution (Figures 2B-2D and Table S1). The expected lipid-headgroup recognition cavity is occupied by a sulfate ion from crystallization solution. Also adsorbed nearby on the protein surface is the sphingoid chain of lysoSM. Notably, the choline headgroup moiety projects outward and away from the protein surface (Figures 2B-D). One phosphate oxygen undergoes hydrogen bonding with the amide nitrogen of Gly144, while the sphingoid base amine hydrogen bonds with Asp60 (Figure 2D). The π-bulge centered at Asp60 (α2 helix) persists in the ACD11/lysoSM complex. At the crystal packing interface of the asymmetric unit, an additional lysoSM molecule is observed (Figure S2A).

ACD11 is a Ceramide-1-phosphate Transfer Protein

Because plants contain no SM and do not produce this sphingolipid, SM transfer by ACD11 was surprising, suggesting that SM serves as a substitute analog for the plant lipid preferred in vivo (Petersen et al., 2008). Also, as noted earlier, the lipid headgroup binding cavity is relatively small, compact, and hood-like (Figure 1B, blue arrow), an arrangement expected to poorly accommodate the bulky SM phosphocholine headgroup. With that in mind, wild-type ACD11 was analyzed for intermembrane transfer of other sphingolipids and phosphoglycerides. A Förster resonance energy transfer approach involving probe lipids with acyl-linked anthrylvinyl fluorophore (AV) enabled testing of lipids with phosphate headgroups, i.e. AV-phosphatidic acid (AV-PA) and AV-ceramide-1-phosphate (AV-C1P), and controls, i.e. AV-galactosylceramide (AV-GalCer), AV-SM and AV-Cer. ACD11 robustly transferred AV-C1P (sphingoid-based) but not AV-PA (glycerol-based) (Figures 2E and 2F). Notably, ACD11 also transferred AV-phyto-C1P (Figures 2E and 2F), as expected by modeling of phyto-C1P docking in the ACD11 binding
site. The sphingoid chains of “phyto” sphingolipid derivatives that predominate in plants lack the 4,5 \textit{trans} double bond but contain a 4-hydroxy group (Markham et al., 2006; Markham et al., 2013). The AV-C1P and AV-phyto-C1P transfer rates depended on protein concentration, required acceptor membranes (Figures 3B, S3A, and S3B), and proceeded at \(~4.5\) C1P and \(~5.6\) phyto-C1P molecules/min/protein. Replacement of phosphate with sugar (AV-GalCer) prevented transfer by ACD11, but not by GLTP. AV-SM transfer by ACD11 was very slow (Figure 2E). The lack of AV-Cer transfer suggested a requirement for phosphate in the head-group for functionality. This was confirmed by competition assays AV-C1P transfer by lipids containing natural hydrocarbon chains (Figure 2G). Only nonfluorescent C1P competed strongly against AV-C1P (Figure S3C). IPC, S1P, lysoSM, and N-hexyl(6:0)-SM (not shown) exerted differing weak competition (Figures 2G and S3D-F). PA and lysoPA minimally slowed the initial AV-C1P transfer rate and were not effective competitors (Figure S3G).

**Crystal Structure of D60N/A-ACD11 with Bound Ceramide-1-phosphate**

Due to the high transfer specificity for C1P, extensive co-crystallization trials were initiated for wt-ACD11 and C1P, but no positive outcome ensued. To achieve success, a point mutation strategy was used to weaken the Asp60-His143 salt-bridge associated with the \(\pi\)-bulge. We focused on Asp60 because mutation of the analogous Asp (D48V) in human GLTP is reasonably well tolerated (Samygina et al., 2011). Asp60 was mutated to residues expected to weaken (Asn) or eliminate (Ala) salt bridging with H143. The D60N-ACD11 mutant maintained \(~25-30\)% activity, while D60A-ACD11 was \(~10-15\)% active compared to wt-ACD11 (Figure 3B).

Both the D60N and D60A mutants yielded crystal complexes with N-dodecanoyl-C1P (12:0-C1P) (Figure 3; Tables S1 and S3) but not with other lipids (e.g. S1P, sphingosine, SM, lysoSM, or PA). In the D60N-ACD11/12:0-C1P crystal complex, the asymmetric unit consists of two ACD11 molecules containing 12:0-C1P bound in two ways (Figures 3C-3F). In one case, both the sphingosine and lauroyl acyl chain of 12:0-C1P are encapsulated in the hydrophobic pocket (Figures 3C and 3E). In the other case, only the lauroyl acyl chain is inserted into the hydrophobic pocket, while the sphingosine chain remains outside the pocket (Figures 3D and 3F). With D60A-ACD11/12:0-C1P complex, a different crystal form was observed involving one protein molecule with bound 12:0-C1P (Figure 3G; Tables S1 and S3). However, the overall structure resembled the sphingosine-out binding mode displayed by D60N-ACD11/12:0-C1P complex (Figures 3G and 3H). Similar sphingosine-out conformers have been observed in human GLTP complexed with glycosphingolipids (Malinina et al., 2006; Samygina et al., 2013; Samygina et al., 2011) and in human CPTP complexed with C1P (Simanshu et al., 2013). Similar positioning of different C1P species occurs in the hydrophobic pockets of ACD11 and
CPTP except for the obvious differences in the bending angle of the outwardly projecting sphingoid chain in the sphingosine-out binding mode (Figure S4). The bending of sphingosine occurs immediately distal to the 4-5 trans double bond where carbon-carbon single bonds exist and torsional rotation is unrestrained, providing conformational optimization for packing at the crystal contact faces of ACD11 and CPTP.

**Recognition of C1P by ACD11**

It is noteworthy that the π-bulge in apo-ACD11 (helix α2) disappears upon binding of C1P in both D60N- and D60A-ACD11, presumably reflecting C1P-induced conformational changes related to portal opening and entry of one or both C1P hydrocarbon chains into the hydrophobic pocket (Figures 3G, 4A and 4B). In both 12:0-C1P conformer complexes with D60N- and D60A-ACD11, the C1P phosphate group is anchored to positively-charged residues on the protein surface via interaction of the three phosphate oxygen atoms with Lys64, Arg99 and Arg103 (Figure 4C). Their functional importance is illustrated by severe reductions in C1P transfer for the K64A, R99E, R99A, and R103A point mutants (Figure 4F). The C1P amide moiety hydrogen bonds with His143 and Asn60. The net effect is disruption of the stabilizing salt-bridge and elimination of the π-bulge characteristic of apo-ACD11.

The hydrophobic pocket that accommodates the sphingosine and acyl chains of C1P is formed by a cluster of nonpolar residues, i.e. Leu, Val, Ile, Phe, Met, Ala and Tyr, that line the two layers of the α-helices in ACD11 (Figure 4D). Insertion and encapsulation of the 12:0-C1P hydrocarbon chains results in disappearance of the intra-helical π-bulge. This π-helix to α-helix structural transition involves large conformational changes for the side-chains of several residues, i.e. Phe47, Phe54, Phe56 and Leu50 (Figure 4E), which move towards the protein surface. This effectively expands the hydrophobic pocket and creates space to accommodate the hydrocarbon chains of 12:0-C1P. Introduction of polarity into the hydrophobic pocket by point mutation (F47Q-ACD11) leads to diminished activity (Figure 4F) affirming the importance of the hydrophobic environment. In the ‘sphingosine-out’ structures, the nonpolar amino acids between helices α5 and α6 interact with the sphingoid chain via hydrophobic and van der Waals interactions enabling adsorption to the protein surface when encapsulation by the hydrophobic pocket does not occur.

**Crystal Structure of D60A-ACD11 in Complex with N-acetyl-C1P**

To define C1P features that contribute to the π-helix-to-α-helix transition needed for C1P chain insertion into the hydrophobic pocket, D60A-ACD11 was co-crystallized with bound N-acetyl(2:0)-C1P (Figures 5A-5E; Tables S1 and S3). As expected, the phosphate headgroup
hydrogen bonds with Lys64, Arg99 and Arg103, but the bidentate hydrogen bonding of Arg99 observed with 12:0-C1P (Figure 4C) is reduced to a single hydrogen bond (Figure 5C). The acetyl chain amide group is unable to hydrogen bond with Ala60 and fails to hydrogen bond with His143 (Figure 5C) leaving the acetyl group on the surface, turned away from Ala60 and outside the hydrophobic pocket. The sphingosine chain also remains outside the hydrophobic pocket (Figures 5B and 5E) adsorbed between helices α5 and α6 on the protein surface. In this altered ‘binding state’, the π-bulge persists suggesting that transitioning of π-helix to α-helix is enhanced when the C1P acyl chain is long enough to enter the hydrophobic pocket (Figure 5D). From the functional standpoint, N-acetyl-C1P competes poorly against AV-C1P transfer by ACD11 as also is the case for S1P (Figure 5F). By contrast, N-acetyl-C1P competes moderately well against CPTP-mediated AV-C1P transfer (Simanshu et al., 2013), and the structure of the CPTP/N-acetyl-C1P complex shows no missing hydrogen bond interactions with the C1P phosphate-amide region, proper engagement of the N-acetyl group in the binding cleft, and encapsulation of the sphingoid chain in the hydrophobic pocket. The differences in position and conformation of bound N-acetyl-C1P molecules in ACD11 and CPTP are shown in Figure S5.

**Perturbations of Sphingolipid Levels in Arabidopsis acd11 Mutants**

To elucidate whether ACD11 involvement in Arabidopsis PCD manifests itself by altering sphingolipid metabolism, sphingolipid levels were profiled in dying leaves of homozygous acd11-1 mutants. An overall accumulation of total sphingolipids (Figure 6A) including long-chain bases (LCBs) (Figure S6A) is evident in acd11 compared to the Landsberg erecta (Ler) wild-type background, with total free ceramides showing the greatest elevation. In plants, the dominant ceramide species (>90%) are phyto-Cers (Markham et al., 2006; Markham et al., 2013) which reportedly are more potent inducers of PCD than Cer (Hwang et al., 2001). To verify and differentiate between effects caused by spontaneous cell death and reduced growth in acd11, we monitored sphingolipid levels upon PCD induction in acd11/NahG plants at 0, 12, 24, 72, and 120 hours after treatment with benzo(1,2,3)thia-diazole-7-carbothioic acid (BTH), a salicylic acid (SA) analog. Introduction of the bacterial transgene NahG into the acd11 background removes endogenous SA needed for development of the cell death phenotype. When acd11/NahG plants are then treated with BTH, cell death is fully reinstated. By 72 and 120 hours after BTH treatment (Figure 6B), a large increase in total Cer is evident compared to Ler and NahG controls, as well as a minor rise in 2-hydroxyceramide, which may reflect free Cer hydroxylation or increased sphingolipid turnover. In contrast, levels of GlcCer and GIPC remain largely unaltered (Figure 6B), suggesting that their increase in acd11 (Figure 6A) is probably due to reduced growth or the dwarf phenotype. Changes in levels of LCB(P), i.e. sphingoid-1-
phosphates, also are insignificant in acd11 (Figure S6B), and thus, may take more time or require stronger inductive conditions (e.g. higher BTH/SA levels) to develop. C1P, which occurs at extremely low levels at normal growth temperature, was not detected. The observed perturbations of sphingolipid levels reveal Cer accumulation during development of acd11 cell death, suggesting that ACD11 mediates Cer synthesis in a SA-dependent manner.

Because cold temperature treatment of Arabidopsis induces substantial and rapid elevation of C1P and LCB(P) by a transduction process regulated by endogenous nitric oxide (Cantrel et al., 2011), the responses of C1P and related sphingolipids to reduced temperature were analyzed in the acd11 background. Figure 7A shows that acd11 loss-of-function results in 3-to 5-fold elevations in the levels of different C1P species of plants subjected to cold treatment. To our knowledge, these are the first quantitative determinations of C1P mass levels in plants, which previously were detected by radiolabeling (Cantrel et al., 2011). Also evident are moderate increases in LCBP, but not LCB (Figure 7B) and dramatic elevations of phyto-Cers (Figure 7C). Interestingly, with phyto-Cer, the palmitoyl (16:0) species and very long acyl species (22-26 carbons) are most affected; whereas, the elevations with C1P are shifted to the moderately long acyl species (16-22 carbons). These changes are consistent with a complex regulatory mechanism involving ACD11.

**DISCUSSION**

**Unique Structural Aspects of the Lipid Headgroup Recognition Center and Hydrophobic Pocket of the ACD11 GLTP-fold**

Despite low sequence homology of ACD11 and other GLTP homologs including human CPTP (Figure S1A), our crystallographic data establish the conserved structural homology shared by Arabidopsis ACD11, human GLTP, and human CPTP (Simanshu et al., 2013) while revealing important differences. Unlike GLTP but like CPTP, ACD11 contains a modified lipid headgroup recognition center that selectively binds C1P, an important signaling lipid linked to cell survival. Comparison of ACD11, GLTP, and CPTP with their preferred bound lipids suggests adaptation and evolutionary conservation of key residues in their GLTP-folds. The net outcome is two divergent subfamilies within the GLTP superfamily. Residues adapted to focus lipid specificity to C1P in ACD11 include Lys64 for Asn52, Arg99 for Leu92, and Arg103 for Trp96 in GLTP. The clustered Lys/Arg residues of ACD11 form a positively-charged triad that is ideally arranged for binding phosphate, explaining the inability of ACD11 to bind sugar headgroups and transfer glycolipids (Petersen et al., 2008). It is noteworthy that Arg103 occupies the same position where Trp acts as a stacking plate for the Cer-linked headgroup sugar in human GLTP, fungal HET-C2, plant GLTP1, and human FAPP2 (Kamlekar et al., 2013; West et al., 2008).
Conversely, residues analogous to Asp60 and His143 of ACD11 are absolutely conserved in the lipid headgroup recognition centers of all known GLTP-folds. The ‘pincher-like’ clamping that occurs when Asp and His hydrogen bond with the Cer moiety amide nitrogen and oxygen ensure a highly conserved and oriented entry of the sphingolipid hydrocarbon chains into the hydrophobic pocket regardless of lipid headgroup composition. The X-ray data rectify earlier 3D homology modeling involving identification of key residues of the ACD11 lipid headgroup recognition center and location of the C-terminus, i.e. Trp206 (Airenne et al., 2006).

A unique feature of the apo-ACD11 GLTP-fold compared to other known GLTP-folds including CPTP is the presence of π-helix, i.e. π-bulge, in helix α2 near the entrance portal of the lipid binding cleft (Figures 1G and 1J). π-bulges exist in only 15% of known proteins but often at locations that enhance/regulate function (Cartailler and Luecke, 2004; Cooley et al., 2010). The π-bulge in apo-ACD11 brings Asp60 and His143 sufficiently close (2.9 Å) to form a salt bridge (Figure 1G), thus providing a potential regulatory mechanism for the ACD11 GLTP-fold. In other GLTP-folds, a water molecule often bridges the Asp and His residues (Figure 1H). In apo-ACD11, the Asp60-His143 salt-bridge created by the π-bulge appears to tightly seal the entry portal region of the hydrophobic pocket (Figure 1G). In D60A-ACD11, the π-bulge persists after binding 2:0-C1P but not 12:0-C1P, suggesting that salt-bridge disruption between Asp60 and His143 by itself is insufficient to induce the π-helix-to-α-helix conformational change needed for the ACD11/C1P complex to become ‘transfer viable’. In addition, the C1P acyl chain needs to be longer than only two carbons. This conclusion is supported by the structure of wt-ACD11 complexed with lysoSM, which has no acyl chain, but displays a bound conformation resembling that of 2:0-C1P in D60A-ACD11 (Figures 5 and S2C). LysoSM is tethered to the surface via its amine group interacting with Asp60, while a sulfate anion occupies the lipid headgroup (phosphate) binding pocket lending credence to the authenticity of the lysoSM binding site. Analogous behavior is observed in human GLTP/hexyl glucoside crystal complexes where the sugar headgroup occupies the glycolipid recognition center despite weak binding affinity and no measureable transfer (Malinina et al., 2006; Zhai et al., 2009). In the ACD11/lysoSM complex, occupation of the phosphate headgroup recognition center by the sulfate anion and the bulky, zwitterionic nature of the phosphocholine lipid headgroup are likely contributors to its minimal interaction and outward projection from the protein (Figure S2A). Similar conformation and surface localization are observed for the 2:0-C1P sphingosine chain in complex with D60A-ACD11 (Figure S2). Thus, despite seemingly adequate positioning on the ACD11 surface, π-bulge persistence renders the lipid binding interaction insufficient to drive robust transfer.
For ACD11 to become fully ‘transfer viable’, uptake of the sphingolipid acyl chain into the hydrophobic pocket and repositioning of specific residues appear to be required. At the molecular level, π-bulge formation at Asp60 results in the Phe56 nonpolar phenyl ring projecting into the hydrophobic pocket to function as a ‘portal gate’ that swings open during lipid acyl chain uptake (Figure 4E). Phe54 orients into the hydrophobic pocket providing conformational stability to apo-ACD11 in the absence of a lipid acyl chain. When C1P contains a sufficiently long acyl chain (e.g. 12:0-C1P), the acyl chain enters deep into the hydrophobic pocket, as shown for D60N-ACD11 and D60A-ACD11. A ‘peristaltic-like shift’ of Ala57 to occupy the position of Phe56 as well as Phe54 being pushed outwards accompanies transformation from π-bulge to α-helix, enabling hydrophobic pocket formation/expansion sufficient to accommodate either one or both hydrocarbon chains of Cer (Figures 4D and 4E). The key role played by Phe56 of helix α2 in functioning as a ‘portal gate’ represents a fundamental difference between the ACD11 GLTP-fold and human GLTP-fold, which uses an ‘oppositely-located’ Phe (Phe148 of helix α6) as the ‘portal gate’ that swings open during hydrocarbon chain insertion (Malinina et al., 2004; Samygina et al., 2011). The global folding topology of ACD11 and conformational adaptability of its flexible, single-cavity, hydrophobic pocket contrast with Cer transfer protein, which uses an α/β fold built around an incomplete U-shaped β-barrel to bind Cer via a START domain lipid cavity (Kudo et al., 2008) (see Supplemental Discussion).

ACD11 Modulates Arabidopsis PCD by Intermediary Regulation of Sphingolipid Levels

The hypersensitive response (HR) in plants generates localized cell death to minimize the spread of pathogens. HR-like PCD also is exhibited by the recessive Arabidopsis acd11 mutant. Despite the known ties between ACD11 and HR-like PCD (Brodersen et al., 2002), determination of the molecular structure and lipid specificity of ACD11 remained unclear until now. Establishment of ACD11 architecture as a C1P-selective GLTP-fold capable of binding/transferring either C1P or phyto-C1P at similar rates provides insights into how this GLTP superfamily member impacts PCD-related processes regulated by key sphingolipid metabolites. While fungal GLTP (HET-C2) and human FAPP2 (C-terminal GLTP-like domain) have both been implicated in PCD-related processes (Fedorova et al., 2005; Paoletti and Clave, 2007; Tritz et al., 2009), no sphingolipid analyses were performed upon in vivo depletion of these glycosylceramide-selective GLTP-folds.

Compared to mammals, plant membranes show fundamental differences in sphingolipid content including large contributions by GlcCer, GIPC, and modified sphingoid chains (e.g. phyto-derivatives) as well as a lack of sphingomyelin and gangliosides (Markham et al., 2006; Markham et al., 2013; Pata et al., 2010). Adjustment to major lipid content during cold
acclimation is well established in plants. Recent studies also show rapid elevations of low level LCB(P)s and C1P (Cantrel et al., 2011). The established signaling functions of such lipids make them leading candidates as early signals during cold acclimation. Because LCB(P)s and C1P content is low under both normal and cold-stressed growth conditions, radiolabeling was initially needed for detection (Cantrel et al., 2011). Determination of LCB(P) and C1P derivative mass levels has been challenging, a situation exacerbated by a dearth of authentic standards (Markham and Jaworski, 2007). This has been especially true for C1P derivatives, which had not been mass quantified in plants until the present study.

Our finding that acd11 deficiency not only alters C1P levels, but also acutely elevates phyto-Cer levels (and LCBP to a lesser extent) establishes a functional link between acd11 expression and sphingolipid metabolic regulation in plants where the dynamic balance between Cer and C1P appears to be critical for regulating PCD (Chen, 2009; Liang et al., 2003; Pata et al., 2010; Reape and McCabe, 2008). While elevated C1P levels induced by acd11 disruption in Arabidopsis also are observed upon RNAi-induced depletion of the ACD11 ortholog CPTP in human cells, it is noteworthy that the dramatic elevations in phyto-Cer levels in acd11 mutants are not duplicated in the Cer levels of CPTP-depleted human cells (Simanshu et al., 2013). This suggests some differing aspects of ACD11 involvement in the regulation of sphingolipid metabolism in plants. Elucidating the mechanistic details and associated kinetics of this involvement will first require detailed analyses of plant sphingolipid metabolic pathways, related regulatory signaling pathways, and changes triggered during cold acclimation.

What is known is that the acd5 mutant lacks Cer kinase (CerK) activity, accumulates Cers, and exhibits PCD (Liang et al., 2003). ACD11 may act in concert with ACD5 (CerK) to maintain the balance of Cer and C1P levels, thus controlling HR-associated PCD. In this context, the loss of IPC synthase activity (erh1 mutant) also results in total Cer accumulation, and both erh1 and acd5 exhibit enhanced HR-associated cell death triggered by the RPW8 resistance protein (Wang et al., 2008). However, Cer accumulation and cell death in acd11, acd5, and erh1 are dependent on the phytohormone, salicylic acid (SA). This suggests that perturbations in sphingolipid metabolism, such as occur in acd11, may regulate SA levels or signaling during R gene mediated HR. Plant R proteins confer recognition of pathogen avirulence proteins and trigger effective innate immune responses (e.g. HR). A genetic screen for suppressors of acd11 cell death (laz mutants) identified the R gene LAZ5. Thus, the absence of ACD11 in acd11 leads to inappropriate HR activation by LAZ5 (Palma et al., 2010). Since sphingolipids are important in both microbial pathogenesis and host defense (Heung et al., 2006), LAZ5 may ‘guard’ ACD11 function(s) in the sphingolipid metabolism that are targeted by
pathogen effectors. Also, transgenic expression of human wt-GLTP and D48V-GLTP (Petersen et al., 2008) suppresses acd11 cell death raising the possibility that the C1P binding/transfer activity of ACD11 is partially dispensable for PCD suppression. This could implicate LAZ5 as a response amplifier that triggers the HR when the local distribution and balance between phyto-Cer and C1P is disturbed, thereby intensifying the response through SA accumulation. Pathogen effector-induced modification or loss-of-function of ACD11 could interfere with normal sphingolipid distribution and trigger a defense response strong enough to deter microbial colonization. Since sphingolipid bases are up-regulated early during an infection or HR, it is probable that sphingolipids are signaling mediators, and not the de facto cell death inducers via membrane perturbations (Mackey et al., 2003; Peer et al., 2010). Testing this hypothesis will require research to clarify the interplay between cellular sphingolipid metabolism and basal immunity in plants.

It’s also possible that loss of ACD11 as a selective carrier blocks C1P exit from the Golgi (or ER) resulting in organelle stress , as occurs in human cells depleted of the ACD11 ortholog, CPTP (Simanshu et al., 2013), and leading to local accumulation of Cer that alters membrane component organization. As ACD11 may be indirectly guarded (Palma et al., 2010), triggering of HR cell death may not rely directly on the absence of ACD11 in the acd11 mutant. In mammals, nonmicrobial ‘danger signals’ instigate obesity-induced inflammation via NLRP3 which senses increasing Cer and induces apoptosis (Vandanmagsar et al., 2011), providing a potential clue as to how the HR might be induced in acd11 by LAZ5 via detection of the accumulation of specific sphingolipid species. Future localization studies on ACD11 and LAZ5 to evaluate possible co-restriction in specific organelles could provide more insights.

EXPERIMENTAL PROCEDURES
Cloning, Expression and Purification
Arabidopsis acd11 ORF (NCBI NP_181016.1) expression in BL21(DE3) pLysS cells using pET-SUMO vector (Invitrogen) enabled Ni\(^{2+}\)-NTA affinity chromatography purification of ACD11 N-terminally-tagged with His\(_6\)-SUMO (see Supplemental Experimental Procedures). Pure proteins were either used for crystallization immediately or flash frozen in liquid N\(_2\) and stored at -80 °C. ACD11 mutants were generated by PCR-based overlap extension and confirmed by DNA sequencing. Expression/purification was the same as for wt-ACD11.

Crystallization
A Mosquito crystallization robot (Molecular Dimensions) was used for initial co-crystallization screening of wt-ACD11 and the D60N and D60A mutants with lysoSM and C1P species (see
Supplemental Experimental Procedures. Positive hits were optimized using hanging drop vapor diffusion method by varying pH and concentration of individual components (Table S3). For data collection, crystals were flash frozen (100 K) in crystallization condition containing 25% (v/v) ethylene glycol. Diffraction datasets were collected on 24-ID-C and 24-ID-E beamlines at the Advanced Photon Source and X29A beamline at National Synchrotron Light Source. Collected datasets were integrated and scaled using the HKL2000 suite (Otwinowski and Minor, 1997). All crystals have different packing interactions leading to different unit cell dimensions and space groups (Table S1).

Structure Determination and Refinement

Ab initio phasing was obtained by soaking apo-ACD11 crystals in 1 mM ethyl mercuric phosphate for 24 h and collecting data (2.45 Å resolution) using a Rigaku RU-H3R X-ray generator equipped with a RAXIS-HTC detector (Table 2). ACD11 structure was determined by the SIRAS method using Hg isomorphous as well as anomalous scattering data, with the 8 Hg sites located and refined for phasing using SHARP (Vonrhein et al., 2007). Apo-ACD11 crystals belonged to space group P2_12_1 and contained four protein molecules per asymmetric unit. The structures of ACD11-sphingolipid complexes were solved by molecular replacement (MOLREP program) using apo-ACD11 (Hg derivative) structure as the search model (Vagin and Teplyakov, 1997) (see Supplemental Experimental Procedures).

ACD11 Intermembrane Lipid Transfer Activity

Förster resonance energy transfer provided kinetic insights into lipid transfer by ACD11. Donor POPC vesicles, containing 1 mole% AV-lipid acylated with (11E)-12-(9-anthryl)-11-dodecenoate and 1.5 mole% 1-acyl-2-[9-(3-perylenoyl)-nonanoyl]-3-sn-glycero-3-phosphocholine [Per-PC] were prepared by rapid ethanol injection (Mattjus et al., 1999). In competition assays, donor vesicles contained 1.5 mol% AV-C1P (Boldyrev et al., 2013) as well as 0.5, 1.0 or 2.0 mole% competitor lipids (Samygina et al., 2011). Both fluorescent lipids were present initially only in donor vesicles where minimal AV emission occurs upon excitation (370 nm) because of energy transfer to Per-PC. ACD11 addition results in an exponential increase in AV emission intensity as the protein transports AV-C1P from donor vesicles (creating separation from the ‘nontransferable’ Per-PC) and delivers to the 10-fold excess POPC acceptor vesicles. The time-dependent increase in AV emission at 425 nm, relative to baseline fluorescence in the absence of ACD11, yields the AV-C1P transfer kinetics (see Supplemental Experimental Procedures).
Plant Material and Sphingolipid Analyses

Arabidopsis acd11 (acd11-1), acd11/NahG and NahG plants in Landsberg erecta (Ler) background have been described (Brodersen et al., 2005). For sphingolipid analyses, plants were grown in soil under short days (8 h light/16 h dark) in chambers at 150 mE/m²s, 21°C and 70% relative humidity. Ler wild type and acd11-1 mutants were grown untreated for 4 weeks before sampling. acd11-1/NahG together with NahG and Ler plants were grown for five weeks prior to spraying with the SA analog BTH (100 µM) and sampling after 0, 12, 24, 72, and 120 hours. Leaf material was harvested from three biological replicates for each genotype and time point. Sphingolipid analysis was performed by mass spectrometry (Bielawski et al., 2009; Markham and Jaworski, 2007). Free and total LCB were analyzed by HPLC after fluorescent derivatization (Bach et al., 2008).

ACCESSION NUMBERS

The atomic coordinates and structure factors for the crystal structures of Arabidopsis wt-ACD11 and mutants in complex with various lipids are deposited in the Protein Data Bank. The accession codes are: apo-ACD11 (4NT1), ACD11/lysoSM (4NT2), D60N-ACD11/12:0-C1P (4NTI), D60A-ACD11/12:0-C1P (4NTG) and D60A-ACD11/2:0-C1P (4NTO).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, three tables, discussion, experimental procedures, and references and can be found with this article online at:

ACKNOWLEDGEMENTS

This research was supported by NIH/NIGMS GM45928 (REB), NIH/NCI CA121493 (DJP & REB), Danish Strategic Research Council 09-067148 (JWM); NSF/MB 0843312 (JEM); NIH/NCRR C06 RR018823 (JB/AB); Spanish Ministerio de Ciencia e Innovacion BFU2010-17711 (LM), Russian Foundation for Basic Research #12-04-00168 (JGM); Hormel Foundation (REB), Abby Rockefeller Mauze Trust (DJP), and Maloris Foundation (DJP). We thank J. Peter Slotte (Åbo Akademi Univ) for the IPC lipid, Helen Pike (UMN-Hormel Institute) for purifying protein used for transfer activity analyses and the staff of X-29 beamline at the National Synchrotron Light Source and ID-24-C/E beamlines at the Advanced Photon Source for help. The Lipidomics Shared Resource, MUSC is partially supported by P30 CA138313, HCC and P20 RR017677, SC COBRE in Lipidomics and Pathobiology.
Author Contributions

DKS: all structural analyses and provided definitive evidence for C1P binding by ACD11; generated all ACD11 point mutants; wrote text. XZ: transfer analyses of wt-ACD11 and ACD11 point mutants; wrote text. DM and DH: prepared *Arabidopsis* mutants for sphingolipidomic analyses; wrote text. JEM: time-based sphingolipidomic analyses on *Arabidopsis* mutants; wrote text. JB/AB: sphingolipidomic analyses on cold-treated *Arabidopsis* mutants. JGM: synthesized fluorescent lipids. LM: contributed to structural data interpretation. JWM: directed set-up of *Arabidopsis* mutant analyses; finalized the write-up. DJP: directed ACD11 structural analyses; finalized the write-up. REB: directed functional and structural analyses, finalized the write-up, coordinated and integrated all section write-ups.
FIGURE LEGENDS

Figure 1. Crystal Structure of Arabidopsis ACD11, Comparison with Human GLTP and CPTP Structures, and π-bulge in ACD11 Lipid-headgroup Binding Pocket.

(A) GLTP-fold of apo-ACD11 (ribbon) showing arrangement of α-helices (cyan) and 3_{10}-helices (lavender).

(B) Structural superposition of ACD11 (cyan) and human GLTP (pink) comparing side-chains (stick representation) involved in lipid headgroup recognition. Blue and red arrows show insertion loop in ACD11 (lavender highlights) and human GLTP (red highlights), respectively, near the lipid headgroup binding pocket.

(C) Structural superposition of ACD11 (cyan) and human CPTP (tan) comparing side-chains (stick representation) involved in phosphate headgroup recognition.

(D) ACD11 surface electrostatics showing positively-charged region around the lipid headgroup binding cavity; blue, positive charge.

(E) Surface electrostatics of human GLTP bound to 18:1 LacCer (stick representation) showing larger, neutral (white) cavity for binding lipid sugar headgroup.

(F) Surface electrostatics of human CPTP bound to C1P (stick representation).

(G) π-bulge centered on Asp60 promotes salt-bridge formation between Asp60 (α2 helix) and His143 (α5-6 loop).

(H) In human apo-GLTP, there is no π-bulge centered on Asp48 and interaction with His140 occurs via water bridging.

(I) In human CPTP, there is no π-bulge centered on Asp56 and no water mediating interaction with His150.

(J) Stereo view of π-bulge in ACD11 α2 helix. Hydrogen bond types $i\rightarrow i-4$ and $i\rightarrow i-5$ are shown as black and pink dashed lines, respectively. Interaction between Asp60 and neighboring His143, Lys64 and a water molecule are shown as green dashed lines.

See also Figures S1, S7 and Tables S1-S3.

Figure 2. Crystal Structure of ACD11 in Complex with Lysosphingomyelin and ACD11 Lipid Transfer Specificity.

(A) Structure of lysosphingomyelin (lysoSM)

(B) Crystal structure of ACD11/lysoSM complex showing ACD11 (ribbon) bound to lysoSM and sulphate (space-filling).

(C) Surface electrostatics of ACD11 in complex with lysoSM.
(D) Lipid headgroup recognition centre residues (lavender) interacting with lysoSM (blue, ball-and-stick) and sulphate. Dashed lines show hydrogen bonds.

(E) Lipid transfer in vitro by Förster resonance energy transfer.

(F) Quantification of initial lipid transfer rates in (E).

(G) Competition against AV-C1P transfer by nonfluorescent lipids. 18:1-C1P competes strongly; lysoSM, moderately; S1P, weakly; and IPC, nearly nil (see Figures S3C-F for kinetic traces) See also Figures S2, S3 and Tables S1 and S3.

Figure 3. Crystal Structures of D60N/A-ACD11 in Complex with 12:0-C1P and Their C1P Transfer Activities.

(A) Structure of 12:0-C1P.

(B) C1P initial transfer rates by wt-ACD11 (red), D60N-ACD11 (green), and D60A-ACD11 (blue) using 3 µg each.

(C) D60N-ACD11 (ribbon) in complex with 12:0-C1P showing the acyl and sphingosine chains both buried in the hydrophobic pocket (sphingosine-in mode; space-filling) in one molecule of the crystal asymmetric unit.

(D) D60N-ACD11 (ribbon) in complex with 12:0-C1P (space-filling) with the acyl chain buried in hydrophobic pocket and the sphingosine chain adsorbing to the protein surface (sphingosine-out mode) in the second molecule of the crystal asymmetric unit.

(E) Surface electrostatics of D60N-ACD11 with bound 12:0-C1P (sphingosine-in mode; ball-and-stick).

(F) Surface electrostatics of D60N-ACD11 with bound 12:0-C1P (sphingosine-out mode).

(G) Structural superposition of C1P headgroup binding pocket of D60N-ACD11 (green) and D60A-ACD11 (yellow) in complex with 12:0-C1P showing disappearance of π-bulge in α2 helix.

(H) Structural comparison of 12:0-C1P binding to D60N-ACD11 (sphingosine-in, magenta and sphingosine-out, green) and to D60A-ACD11 (sphingosine-out, yellow).

See also Figures S4, S7 and Tables S1 and S3.

Figure 4. π-helix Transition to α-helix Induced by C1P Binding in D60N-ACD11 and Mapping of C1P Binding Site.

(A) Stereo representation of α2 helix in the D60N-ACD11/12:0-C1P crystal complex showing absence of π-bulge.
(B) Stereo view of structural superposition of α2 helix observed in apo-ACD11 (cyan) forming a π-bulge and in D60N-ACD11 (salmon) in complex with 12:0-C1P structure without a π-bulge. For clarity, only side chains atoms of Asp60 and His143 are shown.
(C) 12:0-C1P phosphate and amide interactions with D60N-ACD11 residues in the headgroup binding cavity. Hydrogen bonds are shown by dashed lines.
(D) ACD11 residues forming the hydrophobic pocket that accommodates the C1P acyl and sphingosine chains.
(E) Localized conformational changes in apo-ACD11 and D60N-ACD11. Structural superposition of apo-ACD11 and D60N-ACD11/12:0-C1P complex showing residues undergoing large conformational changes during accommodation of C1P hydrocarbon chains. Bound C1P atoms (ball-and-stick) are colored magenta, red, and blue for carbon, oxygen, and nitrogen, respectively. apo-ACD11 and D60N-ACD11 side chains are colored cyan and salmon, respectively.
(F) C1P intervesicular transfer by ACD11 point mutants measured using Förster resonance energy transfer.

Figure 5. Crystal Structure of D60A-ACD11 in Complex with N-acetyl-C1P.
(A) Structure of 2:0-C1P
(B) Crystal structure of D60A-ACD11 (ribbon representation) in complex with 2:0-C1P (space filling) in sphingosine-out conformation.
(C) Inverted (flipped) orientation of lipid amide-acetyl group in sphingosine-out binding mode of 2:0-C1P complexed with D60A-ACD11.
(D) Enlarged view of α2 helix showing π-bulge in the superposed structures of apo-ACD11 and D60A-ACD11 in complex with 2:0-C1P.
(E) Surface electrostatics of D60A-ACD11/2:0-C1P complex (stick representation).
(F) Competition against ACD11-mediated AV-C1P transfer by 2:0-C1P.
See also Figure S5 and Tables S1 and S3.

Figure 6. Constitutive and Inducible Alterations in Sphingolipid Content of acd11 Mutants Grown at Normal Temperature.
(A) Sphingolipids in leaves of dying acd11-1 plants compared to the Ler wild-type control. Data are shown as four sphingolipid classes including phyto- and nonphyto- species (ceramides, Cer; hydroxyceramides, hCer; glucosylceramides, GlcCer; and glycosylinositolphosphorylceramides,
GIPC). Data represent the mean ± SD (n=3) and significant differences from the control are indicated by asterisks (**P<0.01, *P<0.05) based on Students´s t test. dw, dry weight.

**B** Sphingolipid contents in leaves of acd11-1/NahG plants 0, 12, 24, 72, and 120 h after BTH treatment in comparison with Ler NahG and wild-type controls. Plants were grown for five weeks prior to BTH treatment. Sphingolipid contents are shown as in (A). Data represent the mean ± SE (n=3), and letters indicate statistically different groups using one-way ANOVA with groupings by Tukey´s HSD using 95% confidence interval.

See also Figure S6.

**Figure 7. Constitutive and Inducible Alterations in Sphingolipid Content of acd11 Mutants in Response to Cold-Temperature Treatment.**

**A-C** Sphingolipid contents of acd11, acd11-1/NahG, and control plants 72 h after treatment with BTH. Plants were grown for five weeks and subjected to cold (4°C) for 4 h prior to BTH treatment. A) C1P; B) LCB(P); C) PhytoCer. Data represent the mean ± SE (n=3) and significant differences from controls are indicated by asterisks (**P<0.01, *P<0.05) based on Students´s t test.
REFERENCES


Inventory of Supplemental Information

- Supplemental Discussion
- Supplemental Experimental Procedures
- Supplemental References

- **Supplemental Table** (Table S1-S3)
  - Table S1, related to Figures 1, 2, 3 and 5.
  - Table S2, related to Figure 1.
  - Table S3, related to Figures 1, 2, 3 and 5.

- **Supplemental Figures** (Figure S1- S7)
  - Figure S1, related to Figure 1.
  - Figure S2, related to Figures 2 and 5.
  - Figure S3, related to Figure 2.
  - Figure S4, related to Figure 3.
  - Figure S5, related to Figure 5.
  - Figure S6, related to Figure 6.
  - Figure S7, related to Figures 1 and 3.
Arabidopsis accelerated-cell-death11, ACD11, is a ceramide-1-phosphate transfer protein and intermediary regulator of phytoceramide levels


Supplemental Information
SUPPLEMENTAL DISCUSSION

ACD11 Specificity for Ceramide-1-phosphate

Initial testing of ACD11 lipid specificity (Brodersen et al., 2002) occurred prior to structural resolution of the GLTP-fold of human GLTP and mapping of the glycolipid binding site (Malinina et al., 2004). ACD11 showed no transfer of galactosylceramide or ceramide, but limited transfer of sphingosine (monochain sphingolipid). Subsequently, other monochain lipids were observed occupying the GLTP hydrophobic pocket (Airenne et al., 2006; Malinina et al., 2006) and binding weakly to GLTP (Zhai et al., 2009). The weak transfer capability of ACD11 for the dual-chain sphingolipid, sphingomyelin (SM) suggested that SM is a ‘substitute analog’ for the authentic lipid of ACD11 (Petersen et al., 2008) because SM is not produced by plants. Our finding of fast, efficient transfer of C1P by ACD11 solves the long-standing mystery.

π-bulge Occurrence Amongst Lipid Binding/Transfer Proteins

Apo-ACD11 is the first GLTP-fold shown to contain a π-bulge near its lipid binding site. A survey of the Protein Data Bank reveals the existence of π-helix (π-bulges) in only 15% of known proteins but often positioned to enhance/regulate function (Cartailler and Luecke, 2004; Cooley et al., 2010). In other lipid binding/transfer proteins, π-bulges have been observed, i.e. in the START domain of human phosphatidylcholine transfer protein complexed with phosphatidylcholine (PDB ID: 1LN1) (Roderick et al., 2002) and in T cell surface glycoprotein CD1b complexed with phosphatidylinositol (PDB ID: 1GZQ) (Gadola et al., 2002). In both cases, the π-bulge seems to play a role in encapsulation of the lipid by the binding pocket.

Presence of Carboxylated Lysine in the Lipid Headgroup Recognition Center

In two of the four protein molecules present in the asymmetric unit of apo-ACD11 crystal, Lys64 forms a salt-bridge with Asp60 (residue located at the π-bulge) (Figure 1J). In the other two molecules of ACD11 present in the asymmetric unit, additional electron density seen at the tips of Lys64 side chain atoms models as carboxyl groups (carboxylated lysine) based on potential hydrogen bonding to neighboring Arg99 and Arg103 residues (Figures S7C and S7D). Carboxylated lysine has been seen in a number of protein structures and is a protein modification that proceeds nonenzymatically (Li et al., 2005). In most of these proteins, the carboxyl groups of modified lysines function to bridge metal ions that play a structural role in the active site (Dementin et al., 2001; Meulenbroek et al., 2009). In several other proteins, a direct role for carboxylated lysine in their catalytic mechanisms has also been reported (Cha and
Mobashery, 2007). Thus, the presence of carboxylated lysine in the lipid-head group recognition center in ACD11 is consistent with a role for Lys64 in lipid recognition. Point mutation of Lys64 to Ala results in almost complete loss of C1P transfer in in vitro transfer assay (Figure 4F).

**Structural Comparison of ACD11 and CERT**

It is noteworthy that there is a complete lack of structural homology between ACD11 and ceramide transfer protein (CERT). The latter is comprised of a N-terminal pleckstrin homology (PH) domain, a middle coiled domain containing a FFAT motif, and a C-terminal START domain that binds ceramide. In CERT, two helices separated by nine β-strands and two shorter helices form the helix-grip structure that is characteristic of START domains (Kudo et al., 2008). Running through the center of the CERT START domain is a long cavity composed of curved β-sheets and covered by three α-helices and two Ω loops. This cavity is large (~2016 Å³), pre-existing, and amphiphilic, i.e. lined by 26 nonpolar and 10 polar and/or charged residues. Five of the polar/charged residues are buried deep in the cavity where they form hydrogen bonds with the hydroxyl and amide groups of the ceramide headgroup region, while the hydrocarbon chains point back toward the surface. Structural studies of CERT complexed with ceramide species having differing length acyl chains show both the sphingosine and acyl chains are completely buried within the START binding cavity, but that unoccupied cavity space persists near the top of the START cavity when the acyl chain is short (e.g. 6:0 Cer) (Kudo et al., 2008). No extra space exists at the bottom of the START cavity to accommodate a polar group bulkier than the C1 hydroxyl headgroup of ceramide. It is clear that the specific recognition and binding of ceramide by the START domain of CERT differs completely from C1P recognition and binding by ACD11, which contains a positively-charged surface cavity that enables direct access of the bulky, hydrated phosphate headgroup of C1P (Figure 1D). The reversed orientation of ceramide in CERT, with the ceramide headgroup buried deeper than its hydrocarbon chains inside the START binding cavity compared to C1P in ACD11, indicates a fundamentally different mechanism of ceramide uptake and release during membrane interaction compared to ACD11.

**ACD11 Membrane Interaction Site**

Based on previous studies of other GLTP-folds, tryptophan residues projecting from helix α6 appear to play major roles in membrane interaction. In ACD11, Trp145 of helix α6 is expected to function similarly. In this regard, we observed an intriguing arrangement of two ‘extra’ 12:0-C1P molecules at the surface of the crystal complex of D60N-ACD11 containing bound 12:0-C1P. The hydrocarbon chains of the two ‘extra’ 12:0-C1P molecules surround Trp145 (Figures S7A
and S7B) supporting its preference for associating with a lipid environment. Also, the two different conformations of sphingosine observed in C1P molecules bound by ACD11 suggest the likely mode of transfer of C1P from membrane bilayer to the protein involves sphingosine chain entering the hydrophobic pocket last (after the acyl chain) during loading and exiting first during unloading.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cloning, Expression and Purification
Cloning the open reading frame of *Arabidopsis acd11* (NCBI NP_181016.1) into pET-SUMO vector (Invitrogen) enabled heterologous expression of ACD11 N-terminally-tagged with His6-SUMO. Ni2+-NTA affinity chromatography purification from soluble lysate of transformed BL21 (DE3)-pLysS cells (Invitrogen) was followed by overnight cleavage of His6-SUMO tag at 4°C with SUMO protease, Ulp1. Cleanup involved a second nickel-chelating column followed by gel filtration chromatography purification using a HiLoad 16/60 Superdex-75 prep grade column (GE Healthcare) equilibrated with buffer containing 25 mM Tris- HCl, pH 8.0, 100 mM NaCl and 1mM DTT. Peak fractions were pooled and concentrated by centrifugal concentrators to ~20 mg/ml (Vivaspin; 10 kDa cutoff). Protein purity was assessed by SDS-PAGE. Pure proteins were either used for crystallization set-up immediately or flash frozen in liquid N2 and stored at -80 °C. ACD11 mutants were generated by a two-step PCR-based overlap extension method and confirmed by DNA sequencing. Expression/purification was the same as for wt-ACD11.

Crystallization
Apo-ACD11 crystals were grown using the hanging-drop vapor-diffusion method by mixing concentrated protein (20 mg/ml) with an equal volume of crystallization buffer containing 1.2 M potassium sodium tartrate tetrahydrate, 0.1 M Tris (pH 8.5) at 20°C. Small crystals appeared in 2-3 days and grew to full size in 1 week. Initial co-crystallization screening of wt-ACD11 and the D60N and D60A mutants, along with C1P species (2:0, 8:0, 12:0, 16:0 and 24:0 acyl chains) was carried out using a Mosquito crystallization robot (Molecular Dimensions) and involved concentrated protein mixed with phospholipid dispersion (2 mM in 50% ethanol) at ratio 1:0.4 (v/v). Although no wt-ACD11/C1P crystal complexes formed, we did obtain crystals of D60N-ACD11 in complex with 12:0-C1P and of D60A-ACD11 in complex with 2:0- and 12:0-C1P. Co-crystallization with ceramide, sphingosine, sphingomyelin, lysosphingomyelin, and phosphatidic acid was attempted but well-diffracting crystals were obtained only for lysosphingomyelin complexed with wt-ACD11. Lipids were purchased from Avanti Polar Lipids (Alabaster AL). Positive hits were optimized using the hanging drop vapor diffusion method by varying pH and concentration of individual components in the original crystallization condition (Table S3).

Structure Determination and Refinement
Initial attempts to solve ACD11 structure by molecular replacement using known GLTP-like structures as search models were unsuccessful. *Ab initio* SAD phasing using Se anomalous
signal was not possible because of unsuccessful crystallization of L-selenomethionine-derivatized ACD11 despite extensive trials. Successful ab initio phasing was subsequently obtained by soaking apo-ACD11 crystals in 1 mM ethyl mercuric phosphate (EMP) for 24 h and collecting data (2.45 Å resolution) using a Rigaku RU-H3R X-ray generator equipped with a RAXIS-HTC detector. ACD11 structure was determined by the SIRAS method using Hg isomorphous as well as anomalous scattering data, with the 8 Hg sites located and refined for phasing using SHARP (Vonrhein et al., 2007). Apo-ACD11 crystals belonged to space group P2_12_1_2 and contained four protein molecules in the asymmetric unit. Phase improvement with SOLOMON density modification program, implemented in SHARP, produced a clear electron density map (Vonrhein et al., 2007). Structure generation involved several rounds of manual model fitting in COOT and refinement coupled with non-crystallographic symmetry averaging using REFMAC5 (Emsley and Cowtan, 2004; Murshudov et al., 1997).

The structures of ACD11-sphingolipid complexes and apo-ACD11 at high resolution were solved by molecular replacement using the MOLREP program and apo-ACD11 (Hg derivative) structure as the search model (Vagin and Teplyakov, 1997). Model building and refinement for ACD11-complex structures were performed using the COOT and PHENIX.REFINE programs, respectively (Adams et al., 2002; Emsley and Cowtan, 2004). Refinement was monitored with the R_free value calculated for a randomly selected 5-10% of reflections in the data set. During the last stages of model building, lipid molecules were added into the electron density (F_o - F_c map contoured at 2.5 σ) and the complex was further refined. Geometric coordinates and stereochemical libraries for 2:0- and 12:0-C1P and lysoSM were prepared using the Phenix.elbow and PRODRG programs (Moriarty et al., 2009; Schuttelkopf and van Aalten, 2004). Initially, water molecules were added using COOT and then checked manually with electron density and hydrogen bonding interaction with the protein. The final structure was validated using PROCHECK (Laskowski et al., 1993). Statistics for data collection, refinement, and final model geometry are summarized in Tables S1 and S2. Secondary structure elements were assigned using DSSP (http://swift.cmbi.ru.nl/gv/dssp/). Electrostatic surface potentials were calculated with GRASP (Nicholls et al., 1991). Figures were generated using PyMOL (DeLano, 2008).

**ACD11 Lipid Transfer Activity Between Membrane Vesicles**

Förster resonance energy transfer provided kinetic insights into lipid transfer by ACD11. Donor POPC vesicles, containing 1 mole% AV-lipid (anthrylvinyl fluorophore omega-labeled to acyl chain, [(11E)-12-(9-anthryl)-11-dodecenoyl]) and 1.5 mole% 1-acyl-2-[9-(3-perylenoyl)-
nonanoyl]-3-sn-glycero-3-phosphocholine [Per-PC] were prepared by rapid ethanol injection (Mattjus et al., 1999). In competition assays, donor vesicles contained 1.5 mol% AV-C1P (Boldyrev et al., 2013) as well as 0.5, 1.0 or 2.0 mole% competitor lipids (Samygina et al., 2011). Acceptor POPC vesicles were prepared by sonication (Malakhova et al., 2005). Both fluorescent lipids were present initially only in the donor vesicles where minimal AV emission occurs upon excitation (370 nm) because of energy transfer to Per-PC. ACD11 addition results in an exponential increase in AV emission intensity as the protein transports AV-C1P away from the donor vesicles (creating separation from the ‘nontransferable’ Per-PC) and delivers to the 10-fold excess POPC acceptor vesicles. The time-dependent increase in AV emission at 425 nm, relative to the baseline fluorescence in the absence of ACD11, yields the AV-C1P transfer kinetics. Tween 20 detergent addition late in the kinetic time course provides the maximum AV intensity achievable upon ‘infinite’ separation from 3-perylenoyl fluorophore. Maximum transfer, \( \Delta F \), represents the difference in emission intensity in the absence and presence of C1P. The initial lipid transfer rate, \( v_0 \), is obtained by nonlinear regression analyses (ORIGIN 7.0, Northhampton, MA). The standard deviations are calculated at 95% confidence intervals. \( R^2 \) values for all estimates are >0.96.
SUPPLEMENTAL REFERENCES


### Table S1: Crystallographic Data Collection and Refinement Statistics

Values for highest resolution shell are shown in parentheses. Related to Figures 1, 2, 3 and 5.

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<td>8.0 (45.1)</td>
<td>9.4 (68.2)</td>
<td>9.5 (58.6)</td>
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**Refinement**

|                        |                  |              |                     |                     |                   |
| Rwork / Rfree          | 17.4/21.3        | 19.1/22.3    | 19.5/22.7           | 18.2/23.6           | 19.6/24.0         |
| RMSD                   |                  |              |                     |                     |                   |
| Bond lengths (Å)       | 0.006            | 0.004        | 0.003               | 0.008               | 0.008             |
| Bond angles (°)        | 0.968            | 0.770        | 0.760               | 1.10                | 1.09              |
| No. of atoms           |                  |              |                     |                     |                   |
| Protein                | 6222             | 1547         | 3117                | 3079                | 4623              |
| Ligands                | 1                | 88           | 165                 | 76                  | 168               |
| Water                  | 753              | 77           | 45                  | 67                  | 313               |
| Wilson B-factor        | 25.06            | 41.60        | 58.80               | 47.27               | 39.12             |
| Average B-factors      |                  |              |                     |                     |                   |
| Protein                | 26.3             | 40.3         | 47.4                | 52.5                | 40.2              |
| Ligands                | 54.10            | 72.7         | 73.7                | 57.2                | 69.5              |
| Water                  | 35.20            | 46.0         | 43.5                | 49.6                | 45.2              |
| Ramachandran statistics (%) |              |              |                     |                     |                   |
| Mostly allowed regions | 95.7             | 95.6         | 94.3                | 94.8                | 95.0              |
| Allowed regions        | 4.3              | 4.4          | 5.5                 | 5.0                 | 4.8               |
| Generously allowed regions | 0.0             | 0.0          | 0.2                 | 0.2                 | 0.2               |
| Disallowed regions     | 0.0              | 0.0          | 0.0                 | 0.0                 | 0.0               |
Table S2: Data Collection Statistics for ACD11 in Complex with EMP Hg derivative. Values for highest resolution shell are shown in parentheses. Related to Figure 1.

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Figure S1. Sequence Alignments and Structural Comparison for *Arabidopsis* ACD11 and GLTP Homologues. Related to Figure 1.

(A) Sequence alignment of *Arabidopsis* ACD11, human CPTP and GLTP, and fungal HET-C2. Residues conserved across two or more sequences are shaded in black. Similar residues across sequences are shaded in gray. Residues involved in lipid head group recognition are highlighted in lime green (sugar), and blue (phosphate) and red (ceramide amide region).

(B) Structural comparison between *Arabidopsis* ACD11 and human GLTP. Both ACD11 (cyan) and GLTP (pink) are shown in ribbon representation.
Figure S2. LysoSM and N-acetyl-C1P Molecules at Crystal Lattice Interface and in Binding Pocket in ACD11 Crystal Complexes. Related to Figures 2 and 5.

(A) Crystal structure of ACD11 complexed with lysoSM (blue, ball-and-stick) and containing bound sulfate ion. An additional lysoSM molecule (teal, red arrow) localizes at the lattice interface, i.e. crystal packing interface of the asymmetric unit, and interacts via its phosphocholine headgroup with two neighboring symmetry-related ACD11 molecules. The sphingoid chains of both lysoSM molecules stabilize each other on the protein surface via hydrophobic interactions.

(B) Crystal structure of D60A-ACD11 complexed with 2:0-C1P (gray, ball-and-stick). An additional 2:0-C1P molecule (lavender, ball-and-stick; red arrow) is observed at the lattice interface.

(C) Structural superposition of lysoSM/sulfate moiety (ACD11/lysoSM) and of 2:0-C1P (D60A-ACD11/2:0-C1P).
Figure S3. ACD11 Lipid Transfer Specificity Conditions Affecting Transfer of AV-C1P. Related to Figure 2.

(A) Transfer of C1P and phyto-C1P; (B) Requirement for acceptor membranes by ACD11. (C-G) Competition effects by different lipids on AV-C1P transfer by ACD11. Dotted boxes show data regions used to calculate the initial transfer rates summarized in Figure 2G.
Figure S4. Comparison of ACD11 and CPTP structures and bound position/conformation of N-dodecanoyl C1P. Related to Figure 3.

(A) Structural comparison between *Arabidopsis* ACD11-(D60N) and human CPTP bound to 12:0-C1P in sphingosine-in conformation. Both D60N-ACD11 (light magenta) and CPTP (orange) are shown in ribbon representation whereas the bound 12:0-C1P is shown in ball-and-stick representation.

(B) Enlarged view of 12:0-C1P (sphingosine-in conformation) bound to *Arabidopsis* ACD11 and human CPTP as shown in panel A.

(C) Structural superposition of *Arabidopsis* ACD11-(D60N/D60A) and human CPTP bound to 12:0-C1P in sphingosine-out conformation. D60N-ACD11, D60A-ACD11 and CPTP are shown in ribbon representation and colored green, yellow and orange, respectively.

(D) Enlarged view of 12:0-C1P (sphingosine-out conformation) bound to D60N-ACD11, D60A-ACD11 and CPTP as shown in panel C.

(E) Structural superposition of sphingosine-in and sphingosine-out conformations of 12:0-C1P observed in the structures of *Arabidopsis* ACD11 and human CPTP structures. The color coding is the same as in above panels.
Figure S5. Comparison of ACD11 and CPTP structures and bound position/conformation of N-acetyl-C1P. Related to Figure 5.

**A)** Structural comparison between *Arabidopsis* ACD11-(D60N) and human CPTP bound to 2:0-C1P. Both D60N-ACD11 (light magenta) and CPTP (orange) are shown in ribbon representation whereas the bound 2:0-C1P is shown in ball-and-stick representation.

**B)** Enlarged view of 2:0-C1P bound to D60N-ACD11 and CPTP as shown in panel A.
Figure S6. Free LCB Levels upon Constitutive and Inducible Cell Death in acd11 Mutants. Related to Figure 6.

(A) Constitutive cell death; acd11-1 compared to Ler wild-type control.
(B) Inducible cell death; acd11/NahG mutant 0, 12, 24, 72, and 120 hours after BTH treatment compared to Ler NahG and wild-type controls. LCB(P)s are referred to using standard sphingolipid annotation as explained by Markham and Jaworski (Rapid Commun. Mass Spectrom. 2007, 21:1304-14). Data represent the mean of ± SE (n=3).
Figure S7. Role of Trp145 of ACD11 in Membrane Interaction and Carboxylated Lysine-64 Observed in Two Molecules of ACD11 Present in the Asymmetric Unit of apo-ACD11 Structure. Related to Supplemental Discussion involving Figures 1 and 3.

(A) In the crystal structure of D60N-ACD11 complexed with 12:0-C1P, two additional C1P molecules (magenta) are observed at the crystal lattice interface in the vicinity of Trp145.

(B) Stereo view of the region near Trp145 in ACD11. Only partial electron density is observed for one of the two C1P molecules at the lattice interface presumably because of disordering. Side chains of Trp145 are shown in stick representation; and C1P molecules, in ball-and-stick representation.

(C) Helix α2 showing the presence of carboxylated Lys64.

(D) Carboxylated lysine interacts with neighboring arginine residues (Arg99 and Arg103) in the lipid-headgroup recognition center.
Review

Role of autophagy in disease resistance and hypersensitive response-associated cell death

D Hofius1,2, D Munch1, S Bressendorff1, J Mundy1,2 and M Petersen*1

Ancient autophagy pathways are emerging as key defense modules in host eukaryotic cells against microbial pathogens. Apart from actively eliminating intracellular intruders, autophagy is also responsible for cell survival, for example by reducing the deleterious effects of endoplasmic reticulum stress. At the same time, autophagy can contribute to cellular suicide. The concurrent engagement of autophagy in these processes during infection may sometimes mask its contribution to differing pro-survival and pro-death decisions. The importance of autophagy in innate immunity in mammals is well documented, but how autophagy contributes to plant innate immunity and cell death is not that clear. A few research reports have appeared recently to shed light on the roles of autophagy in plant–pathogen interactions and in disease-associated host cell death. We present a first attempt to reconcile the results of this research.

*Corresponding author: M Petersen, Department of Biology, Copenhagen University, Ole Maaloes Vej 5, Copenhagen 2200, Denmark. Tel: membrane vesicle, called the autophagic body, into the lumen fuse with the vacuole/lysosome to release the inner single- and organelles for degradation. Completed autophagosomes (termed phagophores), which enclose cytoplasmic material, are described here. The process is characterized by the formation of programmed cell death.

Autophagy mediates the degradation of bulk proteins and is also involved in the clearance of damaged organelles, insoluble protein aggregates and lipids.1–3 Autophagic digestion and recycling can occur as a survival mechanism to maintain cellular homeostasis and to respond to environmental stresses, such as nutrient depletion or pathogen attack, but may also function as a mediator and/or mechanism of programmed cell death.4–8 Several subtypes of autophagy are described, but macroautophagy (hereafter termed autophagy) is the most extensively studied9 and will be the only form described here. The process is characterized by the formation of large, double-membrane vesicles called autophagosomes. These structures arise from expanding single membranes (termed phagophores), which enclose cytoplasmic material and organelles for degradation. Completed autophagosomes fuse with the vacuole/lysosome to release the inner single-membrane vesicle, called the autophagic body, into the lumen for hydrolytic degradation and recycling.2,10

The mechanism of autophagy is conserved in yeast, plants and metazoans, and involves the action of canonical autophagy related genes (ATG) that synthesize and coordinate membrane rearrangements to allow cellular catabolism.1,2 The core sets of ATG genes seem to be present in all eukaryotes and to be essential for the autophagy pathway (Figure 1). For instance, induction of autophagy requires the negative regulator target of rapamycin (TOR) kinase and the ATG1 kinase complex, which control the activity of the phosphatidylinositol 3-kinase complex containing, for example, ATG6/Beclin1.11 Initiation and completion of autophagosome formation involves two ubiquitin-like conjugation systems to produce ATG12-ATG5 and ATG8-phosphatidylethanolamine (ATG8-PE) conjugates. ATG8-PE conjugation involves the cysteine protease ATG4 and the E1-like protein ATG7, and lipitated ATG8 is linked to and translocated with autophagosomes to the vacuole.12 Therefore, conversion from soluble to lipid bound ATG8, as well as subcellular localization of green fluorescent protein (GFP)-fused protein, have been used to monitor temporal dynamics and spatial regulation of autophagy.13 Finally, recycling and retrieval of autophagy proteins require the ATG9 complex, containing ATG2, ATG9 and ATG18.2,10

A number of excellent reviews provide more details about the molecular mechanisms of autophagy and the individual components required for autophagic complexes and processes7,14–17 (see also Figure 1). In this review, we focus on the role of autophagy in programmed cell death and innate immune responses, with special emphasis on the plant hypersensitive response associated with disease resistance.

Autophagy in Plants

Much has been learned about the requirement for specific ATG genes in the model plant Arabidopsis. Loss-of-function mutations in ATG genes such as ATG7 and ATG5 implicate

Abbreviations: ATG, autophagy related genes; ATG8-PE, ATG8-phosphatidylethanolamine; DAMPs, danger-associated molecular patterns; EDS1, enhanced disease susceptibility1; GFP, green fluorescent protein; HR, hypersensitive response; MAMP, microbial associated molecular patterns; npr1, non expressor of PR genes; PR, pathogenesis-related; R proteins, resistance proteins; SA, salicylic acid; TLRs, toll-like receptors; TMV, tobacco mosaic virus; TOR kinase, target of rapamycin; UDP, uninfected dying tissue

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autophagy as a central player in cellular homeostasis.\textsuperscript{18,19} Processing and delivery of ATG8 to the vacuole under nitrogen-starved condition requires the cysteine protease ATG4 and the ATG12-ATG5 conjugate,\textsuperscript{20,21} and atg5, atg7, atg10, as well as atg12a/b double mutants are hypersensitive to both nitrogen and carbon starvation.\textsuperscript{21–23} Thus, both autophagic-related conjugation pathways seem to be required for autophagy in plants and, as in yeast and other models, the process is required to recycle nutrients during starvation.

Several reports have documented the roles of autophagy in plant development and under stress conditions. During senescence of Arabidopsis leaves kept in darkness (a form of carbon starvation for photosynthetic autotrophs), autophagy seems to be responsible for degradation of the chloroplasts.\textsuperscript{24} and root development also becomes impaired in different atg mutants during nitrogen starvation.\textsuperscript{18,20} Perhaps not surprisingly, autophagy functions in the removal of oxidized proteins during oxidative stress in Arabidopsis.\textsuperscript{25} and downregulation of ATG18a using interference RNA (RNAi) renders plants more sensitive to salt and drought stress.\textsuperscript{26} Collectively, these reports demonstrate that autophagy affects plants in many aspects of their life cycle.

In contrast to autophagy mechanisms in yeast and mammals, information about the signaling pathways triggering the induction of plant autophagy in response to developmental, nutritional and environmental cues is largely lacking. Only recently, direct genetic evidence has been provided that the TOR kinase is a negative regulator of autophagy in higher plants.\textsuperscript{27} Although knockout of the single TOR gene in Arabidopsis proved to be embryo-lethal,\textsuperscript{28,29} knockdown by RNAi resulted in constitutive autophagy under non-stressed conditions in an ATG18-dependent fashion.\textsuperscript{27} In addition, Tap46, the regulatory subunit of protein phosphatase 2A, was recently identified as a downstream effector of the TOR signaling pathway. Depletion of Tap46 reproduced the signature phenotypes of TOR inactivation, including autophagy induction.\textsuperscript{30}

**Autophagy in Immunity**

As autophagy has the ability to eliminate unwanted cellular structures, it is not surprising that this complex and evolutionary ancient pathway also evolved to combat unwanted intracellular microbes. That autophagy could contribute to cellular clearance of microbes was evident already in the 1980s,\textsuperscript{31} but it was first a decade later that the molecular tools to study autophagy in immune responses became available. More recently, autophagy has been shown in a number of cases to contribute to defenses against microbial invasion. For example, autophagy defends mammalian cells against invading Streptococcus.\textsuperscript{32} In contrast to wild type cells, Streptococcus survives and multiplies in ATG5 deficient cells, suggesting that the autophagic machinery is engaged to actively kill the bacteria. These data were supported by micrographs of Streptococci trapped inside autophagossomal structures, and these are absent in ATG5 deficient cells.\textsuperscript{33} Likewise, induction of autophagy suppressed intracellular survival of Mycobacterium tuberculosis in macrophages. In this case, the bacteria are also trapped inside autophagosomal-like structures positive for Beclin1.\textsuperscript{34} Since these reports, a number of excellent reviews have discussed other studies documenting autophagy as an innate defense mechanism for controlling intracellular pathogens in mammals.\textsuperscript{34–36}

In the evolutionary arms race between pathogens and their hosts, some pathogens have also developed mechanisms to avoid or even exploit this defense mechanism to survive and establish infection. Shigella bacteria can escape autophagy
by secreting effectors by means of the type III secretion system. However, mutant bacteria lacking specific effectors become trapped by autophagy during multiplication within host cells and fail to establish infection.\textsuperscript{37} Interestingly, autophagosome-like structures in human cells provide membranous supports for poliovirus RNA replication and, in cells in which autophagy is inhibited via drugs or RNAi against a collection of ATG genes, poliovirus yield is diminished.\textsuperscript{38}

More recently, increased autophagic activity has been linked directly to pathogen surveillance systems in different organisms. For example, mammalian Toll-like receptors (TLRs) detect microbial-associated molecular patterns (MAMP) and induce defense responses upon ligand detection.\textsuperscript{39} Accordingly, stimulation of TLR7 in macrophages leads to increased autophagic activity and elimination of \textit{M. tuberculosis} in an ATG5-dependent manner.\textsuperscript{40} Another example includes the pathogen receptor CD46 that binds Streptococci and triggers autophagy.\textsuperscript{41} Thus, these two examples provide evidence that pathogen recognition in different animal systems is directly linked to higher levels of autophagic activity.

**Autophagy and Cell Death**

Apart from being required to tolerate nutrient deprivation and other stresses, autophagy also represents a cell death pathway conserved across the eukaryotic kingdom. In 2004, Yu \textit{et al.}\textsuperscript{42} reported the requirement for ATG7 and Beclin1 in certain types of cell death in mammalian cell cultures and provided a primary example of autophagic cell death. Since then, numerous reports have argued for or against autophagy as a cell death mechanism, but evidence in favor of autophagic cell death has recently emerged in various genetic models. For example, the conidium of the rice blast fungus \textit{Magnaporthe grisea} undergoes autophagic cell death to establish an infection and, accordingly, \textit{M. oryzae atg8} null-mutants are unable to infect plants.\textsuperscript{43} In Drosophila, physiological cell death of the salivary gland requires the action of \textit{ATG} genes\textsuperscript{44} and autophagy is essential for midgut cell death as well.\textsuperscript{45} In \textit{C. elegans}, necrotic breakdown of neurons is autophagy-dependent and necrotic cell death is accompanied by elevated autophagic activity.\textsuperscript{46} A recent report documented that cell death in the formation of tracheary elements in \textit{Arabidopsis} is inhibited in \textit{atg5} null-mutants and stimulated by increased levels of autophagy.\textsuperscript{47} Such examples indicate that autophagy effectuates cell death pathways critical for many aspects of eukaryotic development (Figure 2).

Beclin1 provides a primary example of a molecular connection between autophagy and apoptosis, an important pathway to cellular destruction in metazoans. Beclin1 is a haplo-insufficient tumor suppressor in mice\textsuperscript{48} involved in the initial formation of autophagosomes. Beclin1 also forms complexes with the anti-apoptotic protein Bcl-2 in mammalian cells\textsuperscript{49} and loss of Beclin1 in \textit{C. elegans} triggers apoptotic cell death.\textsuperscript{50} In \textit{Arabidopsis}, Beclin1 is essential for pollen germination, a feature not associated with other ATG-null mutants,\textsuperscript{51} but knockdown of Beclin1 through antisense or viral-induced gene silencing leads to premature chlorosis and cell death in both \textit{Arabidopsis} and \textit{Nicotiana benthamiana}.\textsuperscript{52,53} It is therefore tempting to speculate that, like Beclin1 in metazoans, plant Beclin1 could also represent a molecular link between autophagy and another cell death route. ATG5 represents an additional molecular link between autophagy and apoptosis, because calpain-mediated cleavage of ATG5 promotes apoptosis through mitochondrial cytochrome C release and caspase activation.\textsuperscript{54} Recently, a conjugate between ATG12 and ATG5 was shown to affect mitochondrial homeostasis and sensitize cells to apoptosis in a context completely separated from the established roles of the ATG proteins in the autophagic pathway.\textsuperscript{55} Collectively, these findings imply that specific \textit{ATG} genes can act as molecular switches and have autophagy-independent functions in homeostatic processes and cell death.

**Autophagy in Plant Immunity and Hypersensitive Cell Death**

Plants rely on a multilayered innate immune system to prevent pathogen invasion and proliferation. Pathogen recognition can occur on the cell surface by pattern recognition receptors that detect MAMPs and induce immune responses such as cell wall thickening and production of antimicrobial proteins.\textsuperscript{56} However, diverse pathogens deliver a variety of virulence determinants, commonly referred to as effectors, into plant cells to evade or suppress MAMP-triggered immunity and to manipulate the host machinery for their own benefit.\textsuperscript{57} In turn, plants have evolved another layer of defense to recognize effector modifications of host target proteins via host surveillance proteins (resistance (R) proteins). These R-mediated defenses often include a localized programmed cell death reaction known as the hypersensitive response (HR) to limit pathogen spread.\textsuperscript{58}

Several examples of the involvement of autophagy in plant immunity and hypersensitive-related cell death have emerged
in the past few years. However, there remains some doubt and apparent contradictions concerning the function(s) of autophagy as a pro-survival or pro-death pathway. In 2005, Liu et al.\textsuperscript{52} linked the activation of autophagy to infection in plants and nicely demonstrated that autophagy contributes to resistance. In addition, they also presented evidence that autophagy was required to restrict the spread of plant hypersensitive cell death, thus functioning as a pro-survival pathway. Activation of the N-resistance gene in \textit{N. benthamiana} by the p50 helicase protein of tobacco mosaic virus (TMV) during infection or upon transient expression triggered hypersensitive cell death, and a few days after local infection, dead cell patches became visible in non-infected tissues on plants silenced for Beclin1.\textsuperscript{52} A similar approach in Arabidopsis supported the observations in \textit{N. benthamiana}, because activation of hypersensitive cell death via the R gene RPM1 upon infection with bacteria also led to macroscopic cell death beyond the infection site in plants silenced for Beclin1, starting roughly 5 days post-infection.\textsuperscript{53} In this context, it should be noted that cell death triggered by RPM1 is executed rapidly and ends after roughly 6–8 h.\textsuperscript{54} This raises the question of whether cell death emerging several days later is directly connected to uncontrolled HR cell death. Nevertheless, the data led to the hypothesis that autophagy prevents unrestricted HR cell death by yet unknown mechanisms and thus functions as a pro-survival pathway in plant–pathogen interactions.\textsuperscript{60,61}

More recently, a pro-death function of autophagy during hypersensitive cell death was reported.\textsuperscript{62} Here, autophagic activity in the infected tissue accompanied the onset of cell death execution triggered by some, but not all types of R proteins. In addition, in cases in which autophagy was induced, cell death was suppressed in local infected tissues in different \textit{atg} mutants. Strongest suppression was found for cell death conditioned by the R proteins RPS4 and RPP1 that signal through the signaling component Enhanced Disease Susceptibility1 (EDS1).\textsuperscript{62} HR cell death triggered by RPM1 was also significantly suppressed in \textit{atg} mutants, but in this case suppression was most prominent in cell death conditioned by the R proteins \textit{RPS4} and \textit{RPP1} in primary infection sites 6 and 12 h post infection in \textit{Arabidopsis}, \textit{atg} mutants (Hofius et al., 2009). HR cell death is retained in younger (3–4 week) \textit{atg} mutants (Hofius et al., 2009; Yoshimoto et al., 2009). HR, Hypersensitive cell death.

In any event, these reports also seem to underscore the importance of the autophagic machinery in limiting pathogen infection in plants. Liu et al.\textsuperscript{52} and co-workers found increased titers of avirulent TMV in infected tissues of Beclin1 silenced \textit{N. benthamiana} plants, and Patel and Dinesh-Kumar\textsuperscript{53} observed increased susceptibility towards virulent strains of \textit{P. syringae} in Beclin1 antisense Arabidopsis plants. Similarly, Hofius et al.\textsuperscript{62} observed increased growth of both virulent \textit{P. syringae} and \textit{Hyaloperonospora arabidopsidis} in different Arabidopsis \textit{atg} mutants. Together, all these findings demonstrate that autophagy can function in plant innate immunity.

Another important contribution comes from Yoshimoto et al.\textsuperscript{67} Primarily scoring macroscopic lesions or visible death in leaves, the authors found no difference in RPM1-triggered cell death beyond the initial infection site in younger \textit{atg} mutants. However, in older \textit{atg} mutants such as \textit{atg5}, they observed lesions in non-infected tissues 6–9 days after infection. Interestingly, these effects were suppressed by removal of the phytohormone salicylic acid (SA) and by mutations in \textit{non expressor of pr genes} (\textit{npr1}).\textsuperscript{67} The authors thus proposed that autophagy negatively regulates cell death by controlling NPR1-dependent SA signaling, although it is unclear why there is a difference between young and old \textit{atg} mutants.

Autophagy-deficient mutants lack the autophagic machinery to remove accumulating cellular ‘garbage’, and in contrast to younger or newly emerged leaves, older \textit{atg} mutant leaves contain higher levels of metabolites, disrupted organelles and oxidized proteins.\textsuperscript{24,25} Such accumulated cellular debris may well disrupt homeostasis, leading to pleiotropic effects.
including accumulation of danger-associated molecular patterns (DAMPs) triggering SA accumulation, and subsequent production of secreted pathogenesis-related (PR) proteins accompanied by ER stress. Autophagy is required to dampen the deleterious effects caused by ER stress and it is well described in other models that atg mutants die upon increased ER stress.69 Thus, accumulated ER stress would be expected to increase the susceptibility of older atg mutants to additional stresses. Interestingly, npr1 has reduced ER stress and expression of PR genes, and mutants like bip2 die upon SA-analog treatment.70 This would explain why npr1 (and SA-deficient mutants) rescue older atg mutants; even in uninfectested tissues, DAMP signals and ER stress-related effects are reduced due to reduced expression of defense genes in npr1/atg5 double mutants. Again, Figure 3 attempts to summarize some of the most important observation done by the different groups.

**Concluding Remarks**

Many questions remain to be addressed on the roles of autophagy in plants. Because autophagy is required for cellular homeostasis, more specific autophagic functions in plant–microbe interactions and immunity are hard to unravel. For example, are plant atg mutants, in contrast to those of other organisms, able to cope with prolonged ER stress and, if not, what is the outcome? Similarly, it may be problematic to use older atg mutants plants, because of pleiotropic effects caused by lifelong accumulation of the kind of cellular ‘garbage’ normally removed by autophagy. In addition, we need to be circumspect in the selection of autophagic mutants amenable for specific studies. For example, the strong chlorotic phenotype of Beclin1 antisense plants suggests that Beclin1 may also be involved in other cell death programs, as is now apparent in metazoans. Moreover, the available collection of atg mutants needs to be further explored to analyze the role of autophagy in MAMP- and effector-triggered immune responses of various host-pathogen systems. Similarly, autophagy components and mechanisms might be specifically targeted by pathogen effector proteins to either suppress defense responses or to promote pathogenicity, for example, of necrotrophic pathogens. Finally, diverse microbial life styles and the changing ‘rules of engagement’ in the evolutionary arms race indicate that autophagy may be co-opted for various purposes by hosts and microbes alike. Therefore, it is possible that host-derived, perimembranal membranes74 are actually autophagic in origin. If so, this could represent an armistice to enable symbiosis for the benefit of both microbes and plants.

**Conflict of interest**

The authors declare no conflict of interest.


