PhD Thesis

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Uncovering the roots of paranoid suicidal plants

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Table of contents

Preface ............................................................................................................................... 3
Acknowledgements ........................................................................................................... 3
Abstract ........................................................................................................................... 4
Resume ............................................................................................................................. 5
Abbreviations .................................................................................................................. 6
Introduction ...................................................................................................................... 7
  The first frontiers in plant immunity ................................................................. 7
  Sensing the enemy – PRRs .................................................................................... 8
  Staging the battle – PTI ......................................................................................... 11
  Changing the field – Effectors ............................................................................. 14
  Return of the plants ............................................................................................... 15
  The last stand – ETI ............................................................................................... 16
  Rearming for another battle – SAR .................................................................... 18
  Bringing out the big guns – Plant NLRs .............................................................. 19
  Taking the pieces apart – NLR structure .............................................................. 20
  Receiving and transmitting – TIR/CC domains .................................................. 21
  Binding it together – NB domain ......................................................................... 23
  The core – P-loop ................................................................................................... 24
  Binding you and binding me – LRR ..................................................................... 26
  NLR function and recognition .......................................................................... 27
  Interacting with the enemy – Direct recognition ............................................... 27
  The guard model – Indirect recognition .............................................................. 28
  Friends and foes – RIN4 ...................................................................................... 31
  Partnering up – NLR pairs .................................................................................... 33
  Two to tango – RPS4/RSS1 ................................................................................ 35
RPS4B/RRS1B

Treasure Your Exceptions – RPM1 and TAO

Integrated decoy/sensor domains

With a little help from my friends – Helper NLRs

Delivering orders – NLR signaling

Changing the program – NLRs and transcriptional reprogramming

The hormonal side of immunity

The art of being in the right place at the right time – NLR localization and mobility

Keeping the troops in line – Regulating of the NLRs

Self-destructive plants – Autoimmune mutants

Suicidal plants – acd11 and LAZ5

Paranoid plants – MPK4, PAT1 and SUMM2

Guarded or not? – camta3 autoimmune mutants

Uncovering the roots of paranoid suicidal plants – Aim of study and concluding remarks

References

Paper 1 – The mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2

Paper 2 – Matching NLR immune receptors to autoimmunity in camta3 mutants using antimorphic NLR alleles

Introduction to FAPP2

References

Paper 3 – Inhibition of autophagy prior to FAPP2 removal restores cargo transfer to the plasma membrane
Preface

This thesis concludes my PhD work at the department of Biology, University of Copenhagen. The main focus of my research has been to reveal the true origin of the camta3 autoimmune phenotypes. This thesis consists of:

(i) A general introduction with focus on NLR functions and consequences of malfunction.
(ii) A research article “The mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2” published in The EMBO Journal.
(iii) A research article “Matching NLR immune receptors to autoimmunity in camta3 mutants using antimorphic NLR alleles”, accepted for publication in Cell Host & Microbe.
(iv) A short introduction to mammalian FAPP2
(v) A draft manuscript “Inhibition of autophagy prior to FAPP2 removal restores cargo transfer to the plasma membrane”.

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Abstract

To evade recognition and to increase their virulence, pathogens deploy effectors into host cells to modify or remove host proteins. Plants can detect these effector mediated changes via Resistance proteins (NLRs). A model thus proposes that NLRs ‘guard’ host ‘guardees’. A corollary to this model is that forms of plant autoimmunity are due to inappropriate NLR activation. We show here that NLR triggered immunity in pat1 can be prevented by the expression of a dominant negative (DN) allele of the NLR SUMM2. We further find that this DN approach is faster than crossing in knock-outs, and has the advantage of ‘poisoning’ redundant genes in cases of NLR homo- and hetero-dimerization. To exploit this, we did a large-scale screen for suppressors of autoimmunity in Arabidopsis and found that camta3 autoimmunity is suppressed by the expression of DN versions of two different NLRs, Dominant Suppressor of CAMTA3 (DSC) 1 & 2. EDS1 levels are elevated in camta3 and CAMTA3 was claimed to negatively regulate immunity through transcriptional regulation of EDS1. In contrast, recent studies provide evidence for CAMTA3 being involved in positive regulation of an early stress response. We report here that EDS1 mRNA levels are no longer elevated in camta3 expressing DSC1-DN or DSC2-DN. This indicates that the camta3 autoimmune phenotype is likely due to inappropriate activation of the NLRs. Interestingly, we found that dsc1 or dsc2 null mutants do not suppress camta3, but camta3/dsc1/dsc2 triple mutants no longer exhibit autoimmunity. We furthermore provided evidence showing that DSC1 interacts with both CAMTA3 and DSC2 suggesting a degree of cooperation between the two NLRs in guarding of CAMTA3. Taken together our data indicate that the increased levels of immune-related transcripts in camta3 are due to NLR activation and not to the loss of CAMTA3 as a transcriptional repressor of immunity.

This thesis comprises an introduction to plant innate immunity with focus on NLRs. The results are presented in three manuscripts. The first, “The mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2” published in EMBO journal, link MPK4 to mRNA decapping and further establishes that the autoimmune phenotype of pat1 is NLR-dependent. The second, “Matching NLR immune receptors to autoimmunity in camta3 mutants using antimorphic NLR alleles”, describing the main focus of my PhD research, is accepted for publication in Cell Host & Microbe. This article establishes camta3 autoimmunity to be NLR dependent and describes a robust systematic method for uncovering the roots of autoimmune mutants. Finally, a draft manuscript entitled “Inhibition of autophagy prior to FAPP2 removal restores cargo transfer to the plasma membrane”, links mammalian FAPP2 to the molecular machinery of autophagy.
Resume

For at undgå at blive genkendt og for at øge deres virulens, indfører patogener effektor molekyler i værtscellel for at ændre eller fjerne værtsproteiner. Planter kan registrere disse effektor medierede ændringer via Resistens proteiner (R-proteiner). En model foreslår således, at R-proteiner bevogter de værts proteiner effektorerne er målrettet mod. En naturlig følge af denne model er, at former for autoimmunitet i planter skyldes unaturlig R-protein aktivering. Vi viser her, at autoimmunitet i pat1 er forårsaget af R-protein aktivering og kan forhindres ved at en dominant negativ (DN) allel af R-proteint SUMM2 udtrykkes. Vi viser endvidere, at brugen af DN R-proteiner er hurtigere end at krydse knock-outs, og at det har den fordel at redundante gener 'forgiftes' i tilfælde af R-protein homono- og hetero-dimerisering. Vi udførte et storstilet overlevels-s-screen for suppressorer af autoimmunitet i Arabidopsis og fandt, at camta3 autoimmunitet undertrykkes i planter der udtrykter dominant negative versioner af to forskellige R-proteiner, Dominant Suppressor of CAMTA3 (DSC) 1 & 2. EDS1 niveauet er forhøjet i camta3 og CAMTA3 er tidligere blevet foreslået som en negativ regulator af immunforsvar gennem transkriptionel regulering af EDS1. I modsætning hertil viser nyere studier bevis for at CAMTA3 er involveret i positiv regulering af et tidligt stress respons. Vi viser her, at EDS1 mRNA-niveauet ikke forhøjet i camta3 planter der udtrykker DSC1-DN eller DSC2-DN. Dette indikerer, at den autoimmune fænotype i camta3 planter sandsynligvis skyldes unaturlig aktivering af disse R-proteiner. Vi viser også at dsc1 eller dsc2 null mutanter ikke undertrykker camta3 fænотyperne, men at camta3/dsc1/dsc2 triple mutanter ikke længere udviser autoimmunitet.

Endvidere dokumenterer vi at DSC1 interagerer med både CAMTA3 og DSC2, hvilket tyder på en vis grad af samarbejde mellem de to R-proteiner i bevogtning af CAMTA3. Vores samlede data indikerer at de øgede niveauer af immunrelaterede gener i camta3 skyldes R-protein aktivering og ikke er en konsekvens af tabt negativ regulering som tidligere foreslået. Denne afhandling består af en introduktion til planters immunforsvar med specielt fokus på R-proteiner. Resultaterne er præsenteret som tre manuskripter. Det første ”The mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2” der er publiceret i EMBO journal, beskriver en sammenhæng mellem MPK4 og mRNA decapping. I dette viser vi også at autoimmunitet i pat1 skyldes unaturlig R-protein aktivering. Det næste manuscript "Matching NLR immune receptors to autoimmunity in camta3 mutants using antimorphic NLR alleles” er hoved fokusset for min PhD afhandling og er for nyligt blevet accepteret til publicering i Cell Host & Microbe. I artiklen beskriver vi at camta3 autoimmunitet skyldes unaturlig aktivering af R-proteiner. Vi præsenterer endvidere vores samling af DN R-gener og beviser at denne screenings metode er overlegen sammenlignet med traditionelle metoder.

Endeligt er der inkluderet et udkast til et manuskript ”Inhibition of autophagy prior to FAPP2 removal restores cargo transfer to the plasma membrane” der forbinder FAPP2 med det molekylære maskineri involveret i autofagi.
Abbreviations

ACD11: ACCELERATED CELL DEATH 11
ADR1: ACTIVATED DISEASE RESISTANCE 1
ATG: AUTOPHAGY-RELATED GENE
ATR1: ARABIDOPSIS THALIANA RECOGNIZED1
BAK1: BRI1-ASSOCIATED RECEPTOR KINASE1
BIK1: BOTRYTIS-INDUCED KINASE 1
CaMV: cauliflower mosaic virus
CAMTA: CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR
CC: coiled-coil
CDPK: calcium dependent protein kinase
CNL: CC-NB-LRR
Col-0: Colombia
DAMP: damage-associated molecular pattern
DND: DEFENSE NO DEATH
DSC: DOMINANT SUPPRESSOR OF CAMTA3
EDS1: ENHANCED DISEASE SUCEPITIBILITY 1
EFR: EF-Tu Receptor
EF-Tu: elongation factor thermos unstable
ET: ethylene
ETI: effector-triggered immunity
ETS: effector-triggered susceptibility
FLS: FLAGELLIN-SENSING 2
GSR: general stress response
HR: hypersensitive response
HSP90: HEAT SHOCK PROTEIN 90
JA: jasmonic acid
LAZ: LAZARUS
LRR: leucine-rich repeats
MAMP: microbial-associated molecular pattern
MAP: Mitogen activated protein
MLA10: MILDEW LOCUS A10
MOS: MODIFIER OF SNC1
MPK: mitogen-activated protein kinase
MPKK: MAP kinase-kinase
MPKKK: MAP kinase-kinase-kinase
MK1: MPK kinase substrate 1
NahG: Naphthalene degradation gene G,
NB: nucleotide-binding
NB-ARC Nucleotide-Binding adaptor shared with
NDR1: NON-RACE SPECIFIC DISEASE RESISTANCE 1
NLR: nucleotide-binding domain leucine-rich repeat domain containing proteins
NOD1: Nucleotide-binding oligomerization domain
NPR1: NON-EXPRESSER OF PR GENES 1
NRG1: N requirement gene 1
NRII: N receptor-interacting protein 1
PAD4: PHYTOALEXIN DEFICIENT 4
PAMP: pathogen-associated molecular pattern
PAT1: Protein Associated with TopoisomeraseI 1
PBS1: AvrPphB susceptible 1
Pip: pipelic acid
PR: PATHOGENESIS-RELATED
PRR: pattern recognition receptor
PTI: PAMP-triggered immunity
RAR1: REQUIRED FOR MLA12 RESISTANCE
RBOH: RESPIRATORY BURST OXIDASE HOMOLOG
RIN4: RPM1 INTERACTING PROTEIN 4
RIPK: RPM1-INDUCED PROTEIN KINASE
RLCK: receptor-like cytoplasmic kinase
RLK: receptor-like kinase
RLP: receptor-like protein
ROC1: ROTAMASE CYCLOPHILIN 1
ROS: reactive oxygen species
RPM1: RESISTANCE TO P. SYRINGAE PV MACULICOLA 1
RPP: RECOGNITION OF PERONOSPORA PARASITICA
RPS: RESISTANT TO P. SYRINGAE
RRS1: RESISTANCE RO RALSTONIA SOLANACEARUM 1
RSRE: rapid stress response element
SA: salicylic acid
SAG101: SENESCENCE-ASSOCIATED GENE 101
SAR: systemic acquired resistance
SERK: somatic embryogenesis receptor kinase
SGT1: SUPPRESSOR OF THE G2 ALLELE OF SKP1
SNC1: SUPPRESSOR OF NPR1-1
SPL6: SQUAMOSA PROMOTER BINDING PROTEIN (SBP)-DOMAIN TRANSCRIPTION FACTOR 6
STAND: signal transduction ATPases with numerous domains
SUMM2: Suppressor of MKK1/MKK2 2
TAO1: TARGET OF AVRB OPERATION1
TCV: Turnip Crinkle Virus
TIP49a: TATA-binding protein (TBP)–interacting protein 49a
TIR: Toll/interleukin-1 receptor/resistance
TMV: tobacco mosaic virus
TNL: TIR-NB-LRR
TPR1: TOPLLESS-RELATED1
T3SS: Type III Secretion System
Ws: wassilewskija
Introduction

The first frontiers in plant immunity

Plants have a sedentary lifestyle and are therefore forced to adapt to their environment in a battle to endure both biotic and abiotic stress factors. Since plants represent a source of nutrients for numerous organisms due to their ability to convert sunlight and carbon dioxide into energy stored as carbohydrates, they are in constant need to fight off damaging invasions (Lodish et al., 2003). If plants cannot defend their resources from being depleted by pathogens and herbivores they cannot thrive. Fungi, bacteria and viruses are worldwide crop pests, which, if not subdued, result in yield losses ranging from significant reduction in quantity and quality to the devastation of harvests (Jones and Dangl, 2006). Depending on their infection and feeding strategies that range from feeding on living host cells to killing plant cells to feed on their contents, phytopathogens are referred to as biotrophic (e.g. the oomycete *Hyaloperonospora arabidopsis*), hemibiotrophic (e.g. the bacterium *Pseudomonas syringae*), or necrotrophic (e.g. the fungus *Botrytis cinerea*) (Kemen and Jones, 2012).

In order to protect themselves from numerous pathogens, plants have developed a multifaceted defense system with a stunning array of constitutive and inducible structural, chemical, and protein-based defenses designed to detect pathogens and stop them before they cause extensive damage (Jones and Dangl, 2006; Katagiri and Tsuda, 2010).

The first frontier of the plant defense is the constitutive defense mechanisms, which include physical barriers such as waxy cuticles and rigid cell walls, as well as constitutively produced antimicrobial compounds (War et al., 2012). These defense components not only function as protection, but many also play important roles in plant physiology. Some provide the plant with strength and rigidity, reduce water loss or protect against UV radiation (War et al., 2012). Overall it is important that the cost of the defense is less than the loss of resources to pathogens to be effective as a survival mechanism. This is in accordance with the dual functions of many of the elements of the constitutive defense (War et al., 2012). Overinvesting in defense in the absence of pathogens can be just as detrimental to survival as disease or lack of proper defense; therefore, it is vital that defense mechanisms are tightly controlled.

The few successful pathogens that breach the preformed barriers often do so by either forced penetration through the epidermis, or entrance through physical injuries or natural openings such as the stomata (Melotto et al., 2008; Toruno et al., 2016). Most plant pathogens are able to invade the
apoplast, instead of entering directly into the host cells, as observed in animal pathogenesis (Melotto et al., 2008). Once inside the plant host, pathogens have to face the inducible immune system before they can proliferate and exploit nutrients from the host.

The first line of inducible defenses is initiated during the early phases of pathogen detection. This initial defense is activated by extracellular pattern recognition receptors (PRRs) that survey the apoplast for conserved immunogenic epitopes or apoplastic pathogen effector proteins (Zipfel, 2014). The immunogenic epitopes are often conserved and functionally important structures, originating directly from invasive pathogens, known as pathogen-associated molecular patterns (PAMPs). PAMPs are typically structures, shared by multiple pathogens, which are essential for function or infection. Examples include the bacterial elongation factor thermos unstable (EF-Tu) and the main proteinaceous component of extracellular flagellum filaments essential for the mobility and ability of bacteria to infect host plants, bacterial flagellin (Felix et al., 1999; Kunze et al., 2004; Meyers et al., 1999; Zipfel et al., 2006). PRRs can also recognize plant-derived compounds that are specifically released by damaged cells. Upon wounding, infection or other stress conditions, plant cells release endogenous elicitors, referred to as damage-associated molecular patterns (DAMPs), such as plant-derived peptides and cell wall fragments (Boller and Felix, 2009; Krol et al., 2010). Activation of PRRs induces convergent intracellular signaling pathways in plant cells, which ultimately initiate defense responses. These outputs culminate in PAMP-triggered immunity (PTI), a complex set of responses intended for resisting pathogen colonization (Boller and Felix, 2009; Jones and Dangl, 2006).

**Sensing the enemy - PRRs**

Most known plant PRRs encode receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Boller and Felix, 2009; Wu and Zhou, 2013). Plant PRRs are functionally analogous to the transmembrane Toll-like receptors (TLRs) of the animal innate immune system (Figure 1). Both plant PRRs and mammalian TLRs, encode membrane-spanning proteins, with extracellular domains able to detect conserved PAMPs (Kawai and Akira, 2009; Maekawa et al., 2011). Additionally, plant and animal innate immune systems recognize an overlapping set of PAMPs. However, although functionally analogous, the organization of protein domains and the downstream molecular players are generally not conserved between plants and animals (Figure 1).
The best-characterized plant PRRs include the bacterial flagellin receptor FLAGELLIN-SENSING 2 (FLS2) and EF-Tu Receptor (EFR) (Boller and Felix, 2009; Kunze et al., 2004; Zipfel et al., 2006). Both FLS2 and EFR are critical to antibacterial immunity. *Arabidopsis thaliana* lines defective in flagellin perception are more susceptible to both adapted and non-adapted strains of the bacterium *P. syringae*, the causal agent of bacterial speck on *Arabidopsis* and tomato (*Solanum lycopersicum*) (Zipfel, 2009). Loss of EFR leads to enhanced susceptibility to *Agrobacterium tumefaciens* (Zipfel et al., 2006) and to weakly virulent strains of *P. syringae* (Zipfel, 2009).

Both FLS2 and EFR are RLKs with intracellular kinase domains, they also both carry large extracellular domains that predominantly consist of leucine rich repeats (LRR) (Bella et al., 2008; Boller and Felix, 2009). FLS2 homologs are found in all higher plants for which genomic information is available (Boller and Felix, 2009). FLS2 recognizes in particular the \(~20\) amino acid epitope flg22, which is relatively conserved across flagellin from diverse Gram-negative bacteria (Boller and Felix, 2009; Felix et al., 1999). Recent studies show that some bacterial strains have acquired mutations that change the shape of their flagellin so that FLS2 can no longer recognize it (Hind et al., 2016). A second flagellin sensor in tomato, FLS3, which detects flgII-28, a region of bacterial flagellin that is distinct from that perceived by FLS2 has recently been described (Hind et al., 2016). The acquisition of the FLS3 receptor may serve as an evolutionary countermeasure to enable tomato to detect bacteria with altered flagellin (Hind et al., 2016). Another well-studied PRR, EFR, which recognizes the N-terminus of bacterial EF-Tu, is only found in the Brassicaceae family (Zipfel et al., 2006). Nonetheless, transgenic expression of *Arabidopsis EFR* in species outside the Brassicaceae family confers to EF-Tu induced immunity. For example, transgenic expression of *Arabidopsis EFR* in the distantly related solanaceous dicot species *Nicotiana benthamiana* and tomato provides broad-spectrum resistance to bacteria belonging to different
genera (Lacombe et al., 2010). This indicates that PRR downstream signaling is well conserved across species and families (Lacombe et al., 2010).

Upon PAMP perception both FLS2 and EFR form ligand-dependent heteromers with the LRR-RLK BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1), which act as a co-receptor and positive regulator (Chinchilla et al., 2007b; Roux et al., 2011). In Arabidopsis and tobacco (Nicotiana tabacum) BAK1 is required for immune responses triggered by multiple PAMPs other than flagellin and EF-Tu, including peptidoglycans, lipopolysaccharides, and cold-shock protein (Chinchilla et al., 2007a; Shan et al., 2008). Thus, to integrate specific PAMP perception into convergent downstream signaling BAK1 appears to associate with multiple PRRs. The plasma membrane-associated receptor-like cytoplasmic kinase BIK1 (BOTRYTIS-INDUCED KINASE 1), is a direct substrate of the FLS2/EFR-BAK1 complexes and phosphorylation of BIK1 is induced upon activation of both FLS2 and EFR (Lu et al., 2010; Zhang et al., 2010). bik1 mutants are compromised in diverse flagellin-mediated responses and immunity to nonpathogenic bacterial infection, underlining the role of BIK1 in intracellular signaling downstream from the PAMP receptor complexes (Lu et al., 2010; Zhang et al., 2010).

BIK1 directly phosphorylates the transmembrane NADPH (nicotinamide adenine dinucleotide phosphate) oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) and positively regulates flg22-triggered calcium influx which can further enhance the activity of the plasma membrane-located RBOHs (Kadota et al., 2014; Li et al., 2014a) (Figure 2). RBOH activation results in an apoplastic burst of reactive oxygen species (ROS). Both ROS- and calcium-dependent signaling pathways play well-established roles during plant immunity. When hindering the RBOHD phosphorylation by BIK1, RBOHD is not activated properly, resulting in increased susceptibility to non-adapted pathogens (Kadota et al., 2014; Li et al., 2014a). Figure 2 shows the events following FLS2 detection of flagellin.

**Figure 2.** Pathogen recognition triggers plant innate immune response. Extracellular recognition and binding of elicitors to the FLS2 receptor enables association with BAK1 leading to phosphorylation of BIK1 and a transient rise in the cytosolic calcium concentration. BIK1 directly phosphorylates RBOHD which leads to the generation of an oxidative burst.
Staging the battle – PTI

Upon PAMP perception, PRRs activate mitogen-activated protein kinase (MPK) cascades. In general, MAP kinase signaling is initiated by activation of a MAP kinase-kinase-kinase (MKKK). MKKK activation, which may be directly or indirectly affected by a PRR, leads to phosphorylation and activation of downstream MAP kinase-kinases (M KKs), which in turn phosphorylate MAP-kinases (MPKs), leading to changes in its subcellular localization and/or phosphorylation of downstream substrates, including transcription factors, and consequently in changed gene expression (Figure 3). In Arabidopsis PAMP perception transiently activate at least two MPK cascades. One is defined by MPKKs MKK4 and MKK5, which act redundantly to activate MPK3 and MPK6 which in turn phosphorylate and activate several downstream pathways leading to transcriptional reprogramming and defense (Asai et al., 2002). Another is defined by the triple kinase MEKK1, which activates MKK1 and MKK2 that in turn phosphorylate MPK4. MPK4 interacts with and phosphorylates MPK kinase substrate 1 (MKS1), leading to activation of the transcription factor WRKY33 (Figure 3) (Bethke et al., 2012; Suarez-Rodriguez et al., 2007).

Figure 3. PRRs activate MPK cascades upon perception of PAMPs. Illustrated is the phosphorylation events involved in MPK signaling during PAMP triggered immunity. MKKKs phosphorylate MKKs, which in turn phosphorylate MPKs. As a result transcriptional reprogramming is initiated.

Loss of BAK1 results susceptibility and marked reduction of flg22- and EF-Tu-triggered activation of MPK3, MPK6, and MPK4 underlining the importance of MPKs in PTI (Roux et al., 2011). Several studies also link MPK regulatory functions to other cellular processes like cellular growth, cell death and senescence, hormone regulation, abiotic stress responses, and autophagy.

Interestingly, MPK4 was recently connected to mRNA decapping, and therefore decay, through its interactions with PAT1 (Protein associated with topoisomerase II), an in eukaryotes important enhancer of decapping (Roux et al., 2015). Eukaryotic mRNA contains stability determinants as a protection against inappropriate degradation (Jiao et al., 2010). mRNA decay is initiated by the removal (decapping) of this protective structures (Garneau et al., 2007; Jiao et al., 2010). That
mRNA translation and degradation play a major role in regulation of gene expression, is especially clear in response to stress when the cells bulk accumulate canonical mRNAs and favor mRNAs required for an appropriate response (Jiao et al., 2010; Munchel et al., 2011; Park et al., 2012). mRNA decapping occurs in processing bodies and upon activation of PTI, these foci increase in number (Roux et al., 2015). The linking of MPK4 to PAT1 and thus mRNA decapping and decay may provide another mechanism by which MPK4 rapidly can regulate immune responses. PAMP perception initiates influx of extracellular calcium into the cytosol followed by activation of calcium dependent protein kinases (CDPKs) (Gao et al., 2014; Lecourieux et al., 2006). The calcium influx signatures are stimulus-specific and are decoded by calcium sensors including calmodulin (CaM) and CDPKs (Lecourieux et al., 2006; Ma et al., 2009). Ca\(^{2+}\) are recognized as major, conserved second messengers in eukaryotic signal transduction and deficiency of the CDPKs leads to impaired defense responses (Gao et al., 2013). Mutations in calcium pumps and channels, necessary for the cytoplasmic influx, including members of the cyclic nucleotide-gated ion channel family, e.g. DEFENSE NO DEATH (DND) 1 and DND2, result in constitutive defense responses (Clough et al., 2000; Jurkowski et al., 2004). This nicely highlights the importance of calcium channels and detectors in plant immune regulation. Both MPKs and CDPKs function to transmit signals within the cell via differential phosphorylation (Asai et al., 2002; Gao et al., 2013). Through this phosphorylation of target proteins, including enzymes and transcription factors, MPKs and CDPKs can control the synthesis and/or signaling of defense hormones, reprogram gene expression, and drive metabolic flow to antimicrobial metabolite synthesis, among other defense responses. CDPKs and MAPKs additionally have versatile roles in the regulation of oxidative burst, cell death, stomatal movements, hormonal signaling, autophagy and transcriptional reprogramming (Gao et al., 2013). Transcriptional reprogramming plays fundamental roles in transducing stress signals and enabling adaptive responses. Plants, like bacteria and yeast, have a variety of stress-specific responses and a rapid and transient general stress response (GSR) (Kultz, 2005). The GSR modulates transcription of an evolutionary conserved suite of stress-responsive genes induced by a wide variety of stresses (Bjornson et al., 2014; Walley et al., 2007). Bioinformatic analyses of promoters of the rapid wound response genes in plants led to the identification of an overrepresented functional cis-element, the *rapid stress response element (RSRE)* (Benn et al., 2014). Further studies showed *RSRE* to have a multi-stress responsive nature. The calmodulin-binding transcriptional activator 3 (CAMTA3) has
been identified as the transcription factor predominantly activating RSRE and by extension inducing the GSR (Benn et al., 2016; Benn et al., 2014; Bjornson et al., 2014).

In order to ensure proper defense activation plants rely greatly on transcriptional reprogramming and therefore transcription factors. Genetic studies have shown a large parallel expansion of the plant transcription factor repertoire, which was found to include several large plant-specific families. This great expansion may be due to their sessile lifestyle and lack of adaptive immunity (Tsuda and Somssich, 2015). Furthermore, studies have found several plant specific transcription factor families that are critical in regulating proper transcriptional responses when plants are challenged with pathogens (Tsuda and Somssich, 2015). One major player in stress specific transcriptional reprogramming during PTI is the family of plant-specific WRKY transcription factors, known for their DNA binding domain with a WRKYGQK sequence motif (Pandey and Somssich, 2009). More than 70% of WRKY genes in Arabidopsis are responsive to pathogen infection (Dong et al., 2003). WRKYs have been shown to bind promoters of defense-related genes, recognizing specific pathogen response elements termed W boxes in the promoter regions. W boxes also exist in the promoter regions of genes encoding plant WRKY transcription factors, indicating the possibility for a feedback loop between different WRKY family members (Dong et al., 2003).

Figure 4 illustrates the complexity of the WRKY regulatory network.

Severe specific WRKYs are required for defense against various pathogens. Examples include WRKY70, which is required for resistance against the oomycete H. arabidopsidis, and WRKY33, which is necessary for defense against infection by B. cinerea and Alternaria brassicicola (Knoth et al., 2007; Tsuda and Somssich, 2015; Zheng et al., 2006). WRKY transcription factors may also act as negative regulators of resistance as defense against the cognate pathogens are enhanced in plants carrying loss-of-function mutations in some WRKYs (Kim et al., 2006).

All in all, PTI consists of a complex set of signaling networks, and activation of these culminates in a number of defense mechanisms, including stomatal closure to limit further pathogen entry, callose...
deposition in the cell wall, production and secretion of antimicrobial compounds such as camalexin and glucosinolate as well as defense related proteins and peptides to inhibit pathogen growth, and restriction of nutrient transfer from cytosol to apoplast limiting bacterial multiplication (Boller and Felix, 2009; Melotto et al., 2008; Wang et al., 2012).

**Changing the field – Effectors**

Once in the apoplast, some pathogens may deliver virulence protein factors, so-called effectors, into the extracellular matrix or into the plant cell. The secreted effectors play dual roles depending on the host genotype. They promote bacterial virulence in susceptible hosts, and elicit immunity in resistant hosts (Jones and Dangl, 2006; Lindeberg et al., 2012; Schreiber et al., 2016). Thus, in susceptible hosts, effector molecules contribute to pathogenesis by disturbing normal physiological and biochemical processes of the host plant. Pathogenic effectors may also repress PTI while changing host cell metabolism to the benefit of the pathogen, allowing the pathogen to further colonize the host (Lindeberg et al., 2012; Mukhtar et al., 2011; Xiang et al., 2008). Plants unable to detect these effectors are susceptible to the pathogen, and the result of infection is effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Large-scale analyses of effector targets have revealed that effectors do not randomly target host proteins. Effectors rather converge upon a limited set of host targets representing for instance key nodes in immune signaling (Mukhtar et al., 2011) (Figure 5). Manipulation of PRR complexes, hormone signaling, and vesicular trafficking are common strategies used by diverse plant pathogens (Lindeberg et al., 2012).

Gram-negative bacterial pathogens, such as *P. syringae*, use a needle-like structure known as the Type III Secretion System (T3SS) for effector delivery (Chang et al., 2014). *P. syringae* is known to deliver ~30 effectors, many of which are capable of suppressing PTI (Lindeberg et al., 2012).

Effectors may interact with and modify components of PAMP recognition mechanisms, such as membrane-receptors and their co-receptors. FLS2 and EFR, as well as their co-receptor BAK1, are targeted by several *P. syringae* effectors to suppress plant immune responses. AvrPto inhibits the kinase activities of FLS2 and EFR to enhance bacterial virulence (Xiang et al., 2008). Both AvrPto and AvrPtoB target BAK1 while AvrPphB cleaves BIK1 to inhibit downstream immune responses (Shan et al., 2008; Zhang et al., 2010).

The plant MPK cascade is also a major target of pathogen effectors. The *P. syringae* HopAI1 effector, a phosphothreonine lyase, is widely conserved in both plant and animal bacterial pathogens, and targets MPK3, MPK6 and MPK4 in *Arabidopsis* (Zhang et al., 2007; Zhang et al.,
HopAI inactivates kinase activities by directly dephosphorylating these kinases. Also the AvrB effector from *P. syringae* targets the kinase MPK4 to promote infection (Zhang et al., 2012). Furthermore, HopF2 inactivates the MPK cascade by ADP-ribosylating MKKs. Plant perception of microbial pathogens involves transcriptional reprogramming toward defense. Transcriptome analyses have revealed that 44% of *Arabidopsis* genes are differentially regulated after inoculation with *P. syringae* (Lewis et al., 2015). In line with this, host transcription factors have been shown to be hubs targeted by multiple pathogen effectors in diverse ways (Mukhtar et al., 2011; Toruno et al., 2016). Effectors can directly target transcription factors or transcriptional repressors by altering their stability and subcellular localization, and by blocking their activity (Toruno et al., 2016). For example, WRKY transcription factors are key in regulating defense responses to several pathogens (Pandey and Somssich, 2009). Therefore, it is not surprising that several effectors have been found to target WRKY transcription factors in different plant species (Sarris et al., 2015). The effector PopP2, expressed by the gram negative bacterium *Ralstonia solanacearum*, is an acetyltransferase that directly targets WRKY transcription factors to dampen immunity (Sarris et al., 2015; Sarris and Jones, 2015). PopP2 uses acetylation to inhibit DNA-binding activities of WRKYs, thereby disabling their transcriptional functions (Le Roux et al., 2015; Tasset et al., 2010). PopP2 selectively target specific WRKYs suggesting that the effector has evolved a degree of substrate discrimination, potentially to avoid negative defense components whose inactivation would be disadvantageous for bacterial infection (Le Roux et al., 2015). A schematic overview of some effector targets are shown in Figure 5.

Figure 5. *P. syringae* injects effectors into the host cell using the T3SS. Pathogen effectors manipulate host immunity on several levels in order to inhibit immunity and enhance infection. Examples of effectors and their targets are marked with red.

Return of the plants

Unperturbed effector action on plant cells would be devastating. Thus it is not surprising that, in a co-evolutionary struggle with their cognate pathogens, plants have developed a second tier of
defense. This is based on cytoplasmic resistance proteins, namely NLRs (nucleotide-binding domain leucine-rich repeat proteins) (Dodds and Rathjen, 2010; Jones and Dangl, 2006; Maekawa et al., 2011). Plant NLRs show structural and functional resemblance to animal NLRs, which are involved in inflammatory and innate immune responses (Duxbury et al., 2016; Jones et al., 2016; Maekawa et al., 2011). One difference, however, is that plant NLRs detects the variable, often host-specific, pathogen effectors, or the activity of these effectors, whereas mammalian NLRs generally intercept intracellular PAMPs or DAMPs to induce immunity (Maekawa et al., 2011; von Moltke et al., 2013). Activated plant NLRs triggers a range of immune responses resulting in initiation of a second layer of defense known as effector-triggered immunity (ETI). ETI is usually quantitatively stronger and longer-lasting than PTI (Cui et al., 2015).

The last stand – ETI

Despite being conceptually two different branches of the plant innate immune system, PTI and ETI do not function as independent, parallel modes of resistance, but are rather intimately linked through shared signaling pathways (Tsuda and Katagiri, 2010; Tsuda et al., 2009). Similar to PTI, NLR activation in ETI leads to diverse cellular changes including sustained Ca\(^{2+}\) influx and subsequent activation of CDPKs, elevated ROS production, MPK activation, enhanced synthesis of defense hormones (including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET)), transcriptional reprogramming, and alteration of endomembrane trafficking (Cui et al., 2015; Spoel and Dong, 2012; Teh and Hofius, 2014).

Transcriptional changes overlap considerably between PTI and ETI (Bozso et al., 2016; Navarro et al., 2004; Tsuda et al., 2009). In tomato, the genes affected by induction of PTI and ETI, overlap by 70% (Bozso et al., 2016). While the signaling networks between PTI and ETI greatly overlap, how the common pathways are used differ (Tsuda et al., 2009). In PTI, signaling mechanisms work synergistically, whereas in ETI, it seems they function independently and work as functional backups for each other (Tsuda et al., 2009). It is further suggested, that quantitative differences in the strength and duration of common pathways produce qualitatively different resistance responses. One model suggests that a key role in ETI is to remove negative constraints on PTI transcriptional programs, thereby amplifying resistance (Cui et al., 2015). This concept is illustrated in Figure 6.
MPKs are normally transiently activated (~5-30 min) during PTI. Recently, however, it was demonstrated that inoculation with *P. syringae* strains expressing the effector AvrRpt2 leads to activation of MPK3 and MPK6 for longer periods ranging from 3 to 10 hours (Tsuda et al., 2013). These quantitative differences in the duration of MPK activation may produce qualitatively distinct transcriptional responses. Activation of this MPK cascades are impaired in plants carrying a loss-of-function mutation in RPS2, the specific NLR that recognize the AvrRpt2 effector. This demonstrates that MPK activation is dependent on, or at least amplified by components of the ETI machinery (Tsuda et al., 2013).

ETI is often, although not always, associated with the hypersensitive response (HR) (Cui et al., 2015). HR is a form of programmed cell death at the site of infection, and was traditionally envisioned to confine and eliminate the invading pathogens. The presence of both an effector and its cognate NLR, overexpression of NLRs, and expression of auto-active NLRs all lead to initiation of HR (Grant et al., 2000; Peart et al., 2005). This clearly illustrates that HR is a consequence of NLR activation. Despite the prevalence of HR during ETI, NLR-mediated cell death responses have in several cases been uncoupled from NLR-mediated pathogen growth restriction and resistance (Clough et al., 2000; Coll et al., 2011; Jurkowski et al., 2004). Studies on dnd mutants showed that these exhibit effector-dependent resistance but do not display HR (Clough et al., 2000; Jurkowski et al., 2004). Thus, HR cell death may occur simply as a consequence of the escalated signaling and the natural rise in toxic intermediates at the infection site. This indicates that HR may be a consequence rather than a cause of resistance (Clough et al., 2000; Coll et al., 2011; Maekawa et al., 2011).

Certain types of HR are dependent on autophagy, a conserved biological degradation process that mediates non-selective, bulk degradation of cytosolic components as well as selective clearance of toxic or redundant structures in eukaryotic cells (Levine and Klionsky, 2004). Autophagy is also known to maintain cellular homeostasis and to improve cellular adaptation in response to various stresses (Levine and Klionsky, 2004; Levine et al., 2011; Mizushima, 2007). In addition to specifically target and degrade a multitude of cellular structures, autophagy furthermore target and

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**Figure 6.** Model of the quantitative variation of immune outputs in PTI and ETI. The same intracellular network results in different relative amplitudes of immune response over time.

*Modified from (Cui et al., 2015)*
degrade invading microbes through specialized cargo receptors (Gomes and Dikic, 2014; Hafren et al., 2017; Levine et al., 2011). In plants evidence for direct pathogen targeting by autophagic cargo receptors are limited, perhaps due to phytobacteria mainly localizing in the apoplastic space. However, autophagy in plants modulates defense responses and thereby influence plant basal resistance to both biotrophic and necrotrophic pathogens (Hofius et al., 2011; Munch et al., 2014; Yoshimoto et al., 2009). Since autophagy both promotes defense-associated hypersensitive cell death induced by avirulent pathogens and restricts unnecessary or disease-associated spread of cell death, the process is playing roles as both pro-death and pro-survival. Effectors from the hemibiotrophic oomycete pathogen, *Phytophthora infestans*, were reported to target autophagy-related processes in plant cells and thus promote autophagosome formation (Dagdas et al., 2016). It is thus likely that pathogens have evolved effector-based strategies to manipulate autophagy pathways to promote virulence.

In mammals, autophagy further has a paramount role in immunity as it directly target viruses and microbes. Mammalian NLRs have been found to recruit and initiate autophagy upon infection (Gomes and Dikic, 2014). In line with this several viruses have evolved measures to antagonize or even exploit host autophagic mechanisms to enhance infection (Chiramel et al., 2013). Recently, it was shown that autophagy in plants also plays both pro- and antiviral roles in the battle between *Arabidopsis* and the cauliflower mosaic virus (CaMV) (Hafren et al 2017). By targeting virus particle forming proteins for degradation, components of the autophagic machinery restrict the establishment of CaMV infection (Hafren et al., 2017). Interestingly, it was also found that the virus trigger autophagy by alternative pathways in order to prevent host tissue death. This extends the timespan of virus production, which greatly enhance the chances for viral transmission (Hafren et al., 2017).

**Rearming for another battle – SAR**

Besides blocking pathogen replication and the spread of infection, ETI can also trigger a general defense response referred to as systemic acquired resistance (SAR) (Mishina and Zeier, 2007). This phenomenon is conserved among diverse plants and confers long-lasting resistance. During SAR systemic tissues are primed for defense, normally from weeks to months, thus protecting the whole plant from secondary infection (Fu and Dong, 2013). Also PTI induces a form of systemic resistance very similar to SAR. Thus, treatment with PTI-inducing *P. syringae* and ETI-inducing AvrRpm1 elicit a highly similar systemic response in *Arabidopsis* (Mishina and Zeier, 2007;
Navarova et al., 2012). SAR generally leads to accumulation of metabolites like the small phenolic hormone SA, and enhanced systemic expression of a variety of classical defense and SAR marker genes such as *PATHOGENESIS-RELATED GENES* 1 (*PR1*), 2 (*PR2*), and 5 (*PR5*) (Mishina and Zeier, 2007; Navarova et al., 2012). SA induces SAR-related gene expression via the downstream regulator NON-EXPRESSER OF PR GENES 1 (NPR1), a transcriptional co-activator and SA receptor (Fu and Dong, 2013). The resulting signal thought not only to include codes for priming of defenses, but also encode detailed information about the primary pathogen infection. Intriguingly, studies on different plant species suggests that the composition of the mobile immune signal in SAR differs depending on the plant species and the type of pathogen conducting the primary infection (Spoel and Dong, 2012).

Cycles of PTI, ETS, and NLR-mediated ETI are major forces shaping plant host-pathogen co-evolution and have led to the so-called zigzag model (Figure 7) (Jones and Dangl, 2006; Maekawa et al., 2011). In this model, pathogen effectors are like double-edged swords, triggering ETI in plants with the corresponding NLRs on the one hand, but exhibiting virulence activities in plants in the absence of the corresponding NLRs on the other hand. This co-evolution of plants and pathogens over millions of years has culminated in large arsenals of immune receptors present in plant genomes (Jones and Dangl, 2006; Toruno et al., 2016).

**Figure 7.** Plants detect PAMPs (red diamonds) via PRRs to trigger PTI. Successful pathogens inject effectors into the host to interfere with PTI and enhance virulence, resulting in ETI. An effector is recognized by host a NLR triggering ETI. This may lead to HR. selective pressure leads to pathogens evolving new or changes effectors, not recognized by the host NLRs. Selection then favors new plant NLR alleles that recognize the modified effector.

*(Jones and Dangl, 2006)*

**Bringing out the big guns – Plant NLRs**

While PAMPs represent conserved microbial molecules, pathogen effectors constitute very divergent groups of fast evolving proteins. Effector recognition therefore requires constant structural and evolutionary adjustment of the corresponding plant surveillance receptors. Genome sequencing of plant species revealed that the repertoire of NLRs in higher plants is generally highly expanded, with approximately 150 known in *Arabidopsis*, compared to ~20 in vertebrates (Cui et al., 2015; Guo et al., 2011; Meyers et al., 2003). Despite their similar biological functions and
protein architecture, comparative genome-wide analyses of NLRs and genes encoding NLR-like proteins suggest that plant and animal NLRs have independently arisen in evolution (Jacob et al., 2013). Their massive expansion renders the NLR family one of the largest and most variable plant protein families (Clark et al., 2007; Jacob et al., 2013). Since plants lack the adaptive immune system known from mammals, which relies on specialized immune cell types, the high number of NLRs found in plants may reflect an important and diverse role for NLRs in plant immunity.

NLR encoding genes are found unevenly distributed in the genome, with many residing in close proximity, forming so-called NLR clusters that contain multiple closely related gene copies or paralogs (Meyers et al., 2003; Zhou et al., 2004). However, NLRs can also exist in single locus with one or multiple functional alleles (Dangl and Jones, 2001). One example of this is seen in flax (Linum usitatissimum), where an individual, yet polymorphic NLR gene encoded at the L locus contains 12 different alleles conferring resistance to different strains of the flax rust fungus Melampsora lini (Ellis et al., 1999).

The molecular mechanisms underlying NLR activation, and how activation is translated into defense responses, have been very challenging to elucidate in plants. Recent reports, however, are beginning to shed some light. It is becoming clear that plant NLRs are targeted to diverse sub-cellular locations, likely depending on where their cognate effectors are detected (Qi and Innes, 2013). Some NLRs even re-localize following effector detection and this re-localization may reflect initiation of compartment specific pathways (Bonardi et al., 2012; Qi and Innes, 2013). However, the more we learn about NLR functions in the plant immune system, the more it becomes clear that not one model will cover the complexity of structure, function and downstream signaling of NLRs.

Taking the pieces apart – NLR structure

NLRs from both plants and animals typically have a modular tri-domain structure with distinct roles for each of the three domains both pre- and post-activation (Jacob et al., 2013) (Figure 8). The majority of NLRs have been found to contain a centrally located nucleotide-binding (NB) domain, a variable number of highly polymorphic C-terminal LRRs, and diverse N-termini (Bonardi et al., 2012). In plants, NLRs can be divided into two major subfamilies, defined by the presence of Toll/interleukin-1 receptor/resistance (TIR) or coiled-coil (CC) motifs in the amino-terminal domain. The central domain of plant NLRs is known as the NB-ARC domain (named after Nucleotide-Binding adaptor shared with APAF-1, plant resistance proteins and CED-4) (Takken et al., 2006). Examples of the classical tri-modular structure of both plant and animal NLRs are
shown in Figure 8. Also an example of a NLR lacking the LRR domain and an example of a NLR with additional C-terminal domain are included; the functions of these domains are described below.

Despite the conserved tri-modular structure, plant NLRs display considerable inter- and intragenic variation and structural diversity, and there are substantial differences in NLRs between species (Chen et al., 2010; Li et al., 2010a). Numerous examples of NLRs with alternative domain architectures, in which the core domains are rearranged or additional domains have been incorporated exists (Sukarta et al., 2016). Furthermore, NLRs with N-terminal TIR domains, which form a major subclass of NLRs in most dicots, are completely missing in monocots (Jacob et al., 2013; Sarris et al., 2016).

### Receiving and transmitting – TIR/CC domains

The NLR N-terminal domains are necessary for signal transduction (Collier and Moffett, 2009; Jacob et al., 2013; Maekawa et al., 2011). Plant NLRs are divided into CC-NB-LRRs (CNLs) which include the well-studied *Arabidopsis* RPM1 (RESISTANCE TO P. SYRINGAE PV MACULICOLA 1), RPS2 (RESISTANT TO P. SYRINGAE) and RPS5 and TIR-NB-LRRs (TNLs), which are homologues to those of the human IL-1R, include tobacco N and *Arabidopsis* RPS4. Although CNLs and TNLs are both involved in immune responses, the two subfamilies are distinct in sequence and signaling pathways, and they cluster separately in phylogenetic analyses (Meyers et al., 1999; Pan et al., 2000).

How these domains relay signals is still not fully understood. However, homo-dimerization has been demonstrated to be important for signaling in most NLRs, and amino acid substitutions hindering dimerization reduce immune responses and HR strength (Bernoux et al., 2011; Maekawa et al., 2011). Both homo- and heterotypic interaction between TIR domains have been found to be implicated in NLR signaling in animals as well as plants (Takeda and Akira, 2005). Recent studies

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**Figure 8.** Examples of the tri-modular structure of plant and animal NLRs. TIR, Toll interleukin-1; CC, coiled coil; CARD, caspase recruitment; NB-ARC/NACHT, nucleotide binding domains; LRR, leucine-rich repeats; WD-40, beta-transducin repeats; WRKY, transcription factor.
further suggest that at least CC-domain homo-dimerization is essential for signaling and HR initiated by NLRs in planta (Casey et al., 2016). Dimerization creates a protein scaffold to which downstream signaling partners bind, a mechanism that has been demonstrated in animal NLRs, but not yet in plant NLRs (Bernoux et al., 2011; Maekawa et al., 2011). Examples of NLRs that self-associate in plants include Arabidopsis RPS5, which has been demonstrated to self-associate prior to activation (Ade et al., 2007). Barley MLA10 forms both monomers and dimers in solution, but CC-domain self-association is required for biological activity (Casey et al., 2016). This correlates with the suggestion that NLRs are found in equilibrium between activated (dimeric) and inactive (monomeric) forms (Bernoux et al., 2016). Recent studies on RRS1 (RECOGNITION OF PERONOSPORA PARASITICA) and RPS4 show that these two NLRs form an inactive heterodimer; upon effector perception the RRS1 TIR domain will release the RSP4 TIR domain, allowing formation of a signaling competent RSP4 TIR homodimer (Williams et al., 2014). Co-expression of RRS1 TIR inhibits RPS4 TIR-mediated cell death, while mutated versions of RRS1 TIR, in which heterodimerization with RPS4 is disrupted, do not (Williams et al., 2014). Mutations at the RPS4 TIR/TIR interface that disrupt the homodimerization abolish RPS4 TIR-induced HR (Williams et al., 2014). Thus RPS4 and RRS1 function as a receptor complex, in which the two components play distinct roles in recognition and signaling, and in which the TIR domains are involved in recognition, signaling, and auto-inhibition, illustrating the complexity of NLR functions in plant immunity.

As shown for RPS4, it is assumed that activation of plant NLRs in general leads to exposure of the N-terminal domain for downstream signaling (Belkhadir et al., 2004b). In line with this hypothesis, overexpression of the N-terminal CC or TIR domains from various plant NLRs causes effector-independent HR (Bernoux et al., 2011; Collier et al., 2011; Maekawa et al., 2011). In contrast, it was shown that overexpression of the CC or NB-ARC domain from RPS5 alone did not induce cell death, while overexpression of a CC-NB construct did, suggesting that the two domains function together to engage downstream components (Ade et al., 2007), yet again underlining the complexity of the NLR functions.

In addition to a role in signaling, the N-terminal domains of plant NLRs can also function in direct or indirect recognition of effectors. RIN4 (RPM1 INTERACTING PROTEIN 4), an important NLR signaling regulator, and PBS1 (AvrPphB susceptible 1), a putative serine-threonine kinase, are both found to directly interact with the N-terminal domains of RPM2 and RPS5 respectively (Mackey et al., 2002). RIN4 and PBS1 are both targeted by effectors which are in turn recognized by the
interacting NLRs (Ade et al., 2007). Modifications of RIN4 or PBS1 monitored by the CC-domains of RPM1 or RPS5, results in initiation of defense responses (Mackey et al., 2002). In addition recognition specificities of flax rust by specific alleles of the flax L locus can be swapped by exchanging only the TIR domains between the alleles (Luck et al., 2000). Contrary to this, substitution of the CC domain of RPS5 with the CC domain of RPS2 did not affect the binding specificity to PBS1. In this case, a full length LRR domain is necessary for RPS5 to recognize PBS1 (Qi et al., 2012). Despite these discrepancies, the N-terminal domains of NLRs are proposed to be responsible for monitoring effector presence, either directly or by effector-induced modifications of their target proteins or, alternatively, by placing the LRR domains in appropriate proximity for optimal surveillance (Belkhadir et al., 2004b). The importance of the TIR domains for NLR function has further been highlighted in several studies showing, that mutations in conserved residues lead to the loss of immunity and/or HR in planta (Dinesh-Kumar et al., 2000; Swiderski et al., 2009).

**Binding it together – NB domain**

The core nucleotide-binding fold in plant NLRs is part of a larger entity called the NB-ARC domain. The NB-ARC domain is a novel signaling motif found in both bacteria and eukaryotes (van Ooijen et al., 2008b). In plants, however, the NB-ARC domain is specific to NLRs (Sarris et al., 2016). The NB-ARC domain is proposed to function as a molecular switch that determines the “on” and “off” state of NLR signaling, with the ADP-bound form for “off” and the ATP-bound form for “on” (Ade et al., 2007; Raidan and Moffett, 2006; Takken et al., 2006; Takken and Tameling, 2009; Tameling et al., 2006) (Figure 9). When in the off state, the NB-ARC domain adopts a closed structure where ADP is preferably bound, and coordinates intramolecular interactions to stabilize the off structure. Upon elicitor perception the NLR activation requires swapping ADP with ATP and adaptation to an open structure of the NB-ARC (Slootweg et al., 2013; Takken and Goverse, 2012) (Figure 9). Recent studies have further shown data supporting a model where the ATP-bound conformation of the NLR has a higher affinity for the elicitor than an ADP-bound conformation, and that cooperation between the TIR and NB-ARC domains plays a regulatory role in the nucleotide exchange. It is also suggested that NLRs are in equilibrium between an ADP- and an ATP-bound state and that the elicitor binds and stabilizes specifically the ATP-bound form, thereby shifting the equilibrium to the on-state (Bernoux et al., 2016). It is generally assumed, and has been demonstrated for a number of NLRs, that binding and hydrolysis of ATP by the NB-ARC domain
induces conformational changes of the overall protein leading to NLR activation (Takken and Goverse, 2012). The conformational changes are believed to further promote homo- or hetero-oligomerization. This in turn will enable the N-terminal domains to engage in downstream signaling.

Numerous conserved motifs, including the Walker B, MHD, and the P-loop have been identified among the NB domains in NLRs (Meyers et al., 1999; Pan et al., 2000). The functional importance of these motifs is exemplified by the many mutations that result in either loss-of-function or auto-activation of the NLRs (Ade et al., 2007; Dinesh-Kumar et al., 2000; Grant et al., 1995; Tameling et al., 2002; Tao et al., 2000). Gain-of-function mutations in the NB domain often lead to hereditary inflammatory diseases in animals and autoimmunity in plants, whereas loss-of-function mutations result in increased disease susceptibility in both kingdoms (Li et al., 2001; Xu et al., 2015).

The core – P-loop

The most conserved motif in the NB subdomain is the P-loop, which is crucial for ATP binding mediated by a conserved lysine residue in the consensus sequence GxxxxGKS/T. Mutations of either the conserved lysine or glycine residues have been shown to result in the abolition of function in numerous plant and mammalian NLRs, by greatly reducing the affinity for ATP binding (Bernoux et al., 2016; Bernoux et al., 2011; Dinesh-Kumar et al., 2000; Inohara et al., 1999; Takken and Goverse, 2012; Tameling et al., 2002). A point mutation of the highly conserved lysine residue of the P-loop of mammalian NOD1 (K208R), well illustrates that the ATP binding is essential for NLR signaling. Direct ligand recognition by NOD1 depends on a functional P-loop and is abolished in the K208R mutants; also the K208R mutation inhibits the ability of NOD1 to induce defense activation (Inohara et al., 1999).

Loss of NLR function arising from mutations within the P-loop is also well documented within plants (Dinesh-Kumar et al., 2000; Tameling et al., 2002; Tao et al., 2000). Point mutations in the

Figure 9. Model for the switch function in NLR activation. In the absence of pathogens NLRs are found in equilibrium between an auto-inhibitory ADP bound state and an ATP-bound primed state. Effector perception results in conformational changes making the NLR prone for ATP binding. Nucleotide exchange triggers additional conformational changes and protein activation. After ATP hydrolysis the NLR returns to the closed off state.
conserved P-loop lysine residue of RPS4, RPS2, and N completely block their function (Dinesh-Kumar et al., 2000; Tao et al., 2000). The tobacco NLR, N, mediates recognition of a helicase domain in the tobacco mosaic virus (TMV) replicase (Caplan et al., 2008b; Hoser et al., 2013). Point mutation in the P-loop of N (Valine -> Asparagine) is dominant negative and completely abolishes its function (Dinesh-Kumar et al., 2000; Mestre and Baulcombe, 2006). Early events in the pathway leading to TMV resistance are oligomerization and stabilization of N. This is abolished when N is mutated in the P-loop (Mestre and Baulcombe, 2006). Since the P-loop motif is buried within the NB subdomain, its impact on protein self-association is likely exerted through conformational changes that accompany nucleotide exchange (Takken and Goverse, 2012). Plants expressing dominant negative N alleles fail to initiate a defense response against TMV infection and exhibit mosaic symptoms similar to those of plants with no N gene.

A point mutation in the P-loop of the Arabidopsis NLR LAZ5 (LAZARUS) also has dominant negative effect (Palma et al., 2010). The laz5-D2 mutation is a transversion changing isoleucine to asparagine, completely abolishing the function of the protein. The dominant laz5D-2 allele was found to suppress acd11 (ACCELERATED CELL DEATH 11) autoimmune phenotypes (Palma et al., 2010). ACD11 encodes a lipid transfer protein able to moderately accelerate the intermembrane transfer of sphingosine and sphingomyelin (Simanshu et al., 2014). Further ACD11 modulates Arabidopsis programmed cell death by intermediary regulation of sphingolipid levels (Simanshu et al., 2014). The laz5-D is likely to interfere with the activity of the wild-type copy of LAZ5, since plants heterozygous for the laz5 null mutation do not suppress acd11. It is thus possible that laz5-D form inactive oligomers with wild-type LAZ5 and/or accessory proteins (Palma et al., 2010).

Besides multiple examples of P-loop mutations in plants being dominant negative (Dinesh-Kumar et al., 2000; Palma et al., 2010; Roberts et al., 2013), examples from Saccharomyces cerevisiae (Shen et al., 1996) as well as Escherichia coli (Wu and Marinus, 1994) have shown similar P-loop mutations to be dominant negative. This underlines a remarkable conservation of the NB-ARC structure and function.

Why the P-loop mutations are dominant negative, is unfortunately not known. One explanation could be that the P-loop mutated proteins through homotypic interactions with wild type proteins could lead to inactive aggregates. It is interesting that in several cases a dominant P-loop mutation exhibit a stronger suppression of NLR-dependent autoimmune phenotypes than a knock out of the NLR does. This is for instance the case for acd11 and LAZ5 (Palma et al., 2010). One explanation for this is that expression of the DN alleles inhibits the function of NLRs working in complex. Thus
P-loop mutations can possibly poison NLR complexes and for this reason may be superior in uncovering NLR functions in autoimmunity since NLR redundancy will cease to be an issue.

**Binding you and binding me – LRR**

LRR domains are versatile binding motifs found in thousands of proteins in diverse species, ranging from viruses to eukaryotes (Bella et al., 2008). The LRR domain in plant NLRs has been implicated in direct pathogen effector recognition (Qi and Innes, 2013). However, direct interaction between NLR proteins and effectors has been demonstrated for only a subset of plant NLRs. One example is the race-specific interactions between the *Arabidopsis* NLR RPP1 LRR domain and the effector ATR1 (ARABIDOPSIS THALIANA RECOGNIZED1), expressed by *H. arabidopsidis* (Krasileva et al., 2010). Exchange studies of the flax *L* alleles showed that several of the repeats of the LRR contribute to resistance specificity. Recognition specificity can be swapped by exchanging the LRR domains between alleles in the *L* and *R* loci of flax (Dodds et al., 2001; Ellis et al., 1999). Removal or substitution of LRR domains can result in loss of sensitivity to cognate pathogen effectors demonstrating the importance of this domain in effector recognition (Rairdan and Moffett, 2006; van Ooijen et al., 2008a). The LRR domains are the most polymorphic part of plant NLR proteins, which likely reflects that this domain in particular has been coevolving with pathogen effectors and as a result is under massive diversifying selection (Chen et al., 2010; Clark et al., 2007; McDowell et al., 1998; Qi and Innes, 2013).

In addition to a role in effector recognition, the LRR domain also plays an important role in keeping NLRs in the “off” state. Consistent with the intramolecular interactions necessary for an auto-inhibitory role, the LRR domains of several NLRs have been shown to co-immunoprecipitate with separately expressed NB-ARC domains (Ade et al., 2007; Moffett et al., 2002; Rairdan and Moffett, 2006; van Ooijen et al., 2008b). This is illustrated by RPS5 where the LRR domain physically associates with the NB-ARC domain (Moffett et al., 2002). Furthermore, the removal of the LRR domain can, in some cases, lead to autoimmunity and overexpression of RPS5 lacking the LRR domain induces HR, whereas overexpression of the full length RPS5 does not (Ade et al., 2007). Auto-activation is also frequently observed when LRR domains are swapped between NLR proteins. Replacement of the LRR domain of RPS4 with the domain from RPS2 leads to auto-activity, suggesting that the LRR and NB-ARC domains have co-evolved (Qi et al., 2012; Rairdan and Moffett, 2006). Altogether this underlines the importance of the auto-regulatory role of the LRR domain (Ade et al., 2007).
NLR function and recognition

The modes by which NLRs recognize pathogen effectors are diverse. Some NLRs interact directly with the pathogen effectors (Dodds et al., 2006; Krasileva et al., 2010). Other NLRs monitor host proteins that are targeted by pathogen effectors (Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008). Yet others function in NLR pairs, where each partner has a specific role in effector perception and defense activation (Cesari et al., 2014b; Williams et al., 2014). In some cases transcription of NLRs can even be activated directly by effectors with transcription factor functions (Khan et al., 2016). This may be an example of the pathogen-host co-evolutionary competition at a transcriptional level. The effectors act as transcription factors binding to promoters of desired host proteins. The host has in turn evolved to exploit this strategy to trigger NLR expression regulated by the promoter in mention (Caplan et al., 2008a; Khan et al., 2016).

The two major classical modes of effector perception utilized by plant NLRs are, as already mentioned, either direct or indirect recognition. In case of the direct recognition, an effector is detected by direct physical interaction with its cognate NLR. During the indirect recognition, a NLR senses modifications of self, caused by the effector (Jones and Dangl, 2006). Indirect recognition enables a single NLR to recognize multiple effectors, irrespective of effector structures, when effectors target the same host protein or pathway (Chisholm et al., 2006; Jones and Dangl, 2006). Recently, it was demonstrated that some NLRs can further detect several sequence-unrelated effectors by direct binding (Cesari et al., 2013).

Interacting with the enemy – Direct recognition

Early research in plant pathology characterized interaction between plants and pathogens as a gene-for-gene relationship in which there for every NLR gene in plants is a matching pathogen avirulence gene. In this model the outcome of the plant-pathogen interaction is determined by whether a pathogen effector coincides with a corresponding plant NLR (Flor, 1971). If no cognate NLR is present in the plant, the result will be ETS. Conversely, if the cognate NLR is present it will initiate ETI upon recognition of the effector (Figure 10).
Direct recognition of effectors is seen in Arabidopsis where, as mentioned above, NLRs encoded by some RPP1 alleles have been shown to directly bind the oomycete effector ATR1 (Steinbrenner et al., 2015). Different ATR1 alleles from various H. arabidopsidis strains are only recognized when matched with distinct alleles at the RPP1 locus, suggesting that the recognition is race-specific (Krasileva et al., 2010). Chimeric exchanges between RPP1 alleles mapped distinct interacting surfaces of the LRR domain contributing to this allele-specific recognition of the ATR1 effector (Steinbrenner et al., 2015).

The gene-for-gene model, however, has some discrepancies. Considering the large numbers of pathogen molecules that are delivered into plant cells, direct recognition of effectors by a limited number of plant NLR immune receptors is not sufficient to explain how plants can effectively fight multiple pathogens. Based on experimental data, different models involving indirect recognition have been proposed, including the “guard model” and the “decoy model” (Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008; Wu et al., 2015). In these models, NLRs detect the biochemical activity of effectors, rather than the effector epitopes (Jones and Dangl, 2006). A yeast-two-hybrid screen of Arabidopsis-derived immune proteins and effectors from P. syringae found that merely ~6% of the NLR fragments tested interact directly with a pathogen effector. For comparison ~50% of the NLR interactors were known effector targets (Mukhtar et al., 2011), supporting indirect recognition as the most pronounced mode of sensing pathogens in plants. This indirect recognition reduces the number of NLRs a plant has to deploy to protect against a potentially vast number of structurally diverse and rapidly evolving effectors (Chisholm et al., 2006; Jones and Dangl, 2006) and illustrates a higher level of complexity in pathogen recognition by NLRs.

The guard model – Indirect recognition

Indirect recognition of effectors is often described by the guard model. This model proposes that NLRs (the guards) detect the effectors by monitoring changes in the effector targets (the guardees) (Dangl and Jones, 2001). The concept is illustrated in Figure 11. The guardee itself is often required

![Figure 10. Direct effector recognition. NLRs are kept in an autoinhibitory off state. Upon effector perception the ADP is swapped to ATP leading to conformational changes in the NLR. The active NLR triggers ETI. However, if the effector is not recognized the result is ETS.](Image)
for virulence of the effector protein when not sensed by an NLR (van der Hoorn and Kamoun, 2008). An expansion of this model is that the host protein monitored may be either the operational target of the effector and in this case it is called a guardee, since it is guarded by the NB-LRR, or a mimic of the operational target in which case it is called a decoy (Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008). Unlike guardees, the absence of decoys does not affect pathogenicity of an effector (van der Hoorn and Kamoun, 2008). Modifications of decoys by effectors, however, are perceived by matching NLRs, leading to ETI (van der Hoorn and Kamoun, 2008; Zipfel and Rathjen, 2008). Decoys and guardees are in some cases a direct part of the NLRs, as integrated domains. This phenomenon is discussed below.

The classical guard model originally implied a one-on-one matching relationship of effector, guardee, and guard NLR (Dangl and Jones, 2001). However, one guardee can be targeted by multiple effectors with different origins or different biochemical properties. Furthermore, modifications of a guardee can be recognized by multiple host NLRs and one NLR can even monitor multiple guardees to maximize surveillance capacity (Axtell and Staskawicz, 2003; Wang et al., 2015; Wilton and Desveaux, 2010). Today several variations of the guard model exist. NLRs can either be constitutively bound to the guardee, which is the case for RIN4 and its guards RPM1 and RPS2, this is described in depth below. Alternatively, the NLR may bind the guardee only after its interaction with the effector (Caplan et al., 2008b). The tobacco NLR N only interacts with its guardee, NRIP1 (N receptor-interacting protein 1), when the p50 from TMV is present (Caplan et al., 2008b).

The Arabidopsis CNL RPS5 detects the presence of AvrPphB by monitoring the integrity of PBS1 (DeYoung et al., 2012). Insertion of a few amino acids at the AvrPphB cleavage site of PBS1 activates RPS5 as strongly as PBS1 cleavage does, suggesting that RPS5 senses even relatively subtle conformational changes in PBS1 associated with its cleavage (DeYoung et al., 2012). Recently the recognition specificity of RPS5 was successfully altered by exchanging the AvrPphB cleavage site in PBS1 with the cleavage sequence targeted by unrelated pathogen-secreted proteases
This nicely illustrates that RPS5 is activated by the PBS1 modification independent of the cause of the modification. AvrPphB inhibits PTI by cleaving PBS1-like kinases, like BIK1, which often are important in immune signaling (Zhang et al., 2010). This suggests that these kinases may be the real virulence targets and that PBS1, which does not directly contribute to host immunity, has evolved as a decoy (Schreiber et al., 2016). RPM1 can also be activated by mutations mimicking effector action in its guardee RIN4 (Chung et al., 2011; Li et al., 2014b; Liu et al., 2011). RPM1 and RPS5 not only recognize specific modifications of their guardee, but also seem able to activate upon mutations in regions surrounding the effector-altered residue (Chiang and Coaker, 2015; Li et al., 2014b). This indicates that NLRs may be able to perceive general disruption of guarded proteins, which could enable recognition by one guard of diverse effectors with similar host targets (Chiang and Coaker, 2015).

In a recent study Xu et al. found that the human non-NLR disease resistance protein Pyrin senses bacterial modifications of Rho GTPases (Xu et al., 2014). Pyrin detects pathogen virulence activity rather than directly recognizing microbial products, which is how most mammalian NLRs have been found to work so far (Xu et al., 2014). Also the human NLR NOD1 is capable of sensing the activity of Salmonella enterica effector SopE, rather than the pathogen itself (Duxbury et al., 2016). This suggests that the guard/guardee model may be relevant in mammalian systems as well. However, the evidence so far for the indirect guard mechanism as a general means for NLR recognition is weaker in animals than in plants (Duxbury et al., 2016). Recently it was furthermore suggested that NLRs could be monitoring perturbations in cytoplasmic homeostasis rather than specific guardee proteins (Liston and Masters, 2017). This is supported by emerging evidence showing that the inflammasome components NLRP3 and pyrin do not directly detect molecular patterns but rather seem to detect homeostasis altering molecular processes (Liston and Masters, 2017). Direct evidence for this homeostasis surveillance model, however, still needs to be presented.

Monitoring guardees does provide a potent flexibility to the innate immune system regards to detecting evolutionarily novel infections. On the other side, perceiving changed self rather than directly detecting the presence of pathogens, escalates the risk of inappropriate inflammation and autoimmune reactions. This is known from plants where several examples of autoimmunity have been reported to be caused by inappropriate NLR activation due to mutations in guardees (Palma et al., 2010; Roux et al., 2015). It is thus also distinctly possible that mammalian autoimmune diseases could be due to deficiencies in guardees leading to inappropriate inflammation.
Friends and foes – RIN4

The probably most studied guardee is the Arabidopsis RIN4. RIN4 is a widely conserved and fairly abundant plasma membrane tethered protein. Genomes for all land plants sequenced to date encode RIN4 orthologs (Chung et al., 2014). RIN4 is also found fused to NLRs as an integrated decoy/sensor in several species (Sarris et al., 2016). RIN4 regulates PTI and has recently been described as a phosphor-switch (Chung et al., 2014). Phosphorylation of RIN4 serine 14, following activation of the PRRs FLS2 or EFR, enhances PTI responses (Chung et al., 2014). Furthermore, RIN4 has been demonstrated to be an important regulator of NLR signaling and ETI induction in Arabidopsis, tomato, soybean (Glycine max) and lettuce (Lactuca sativa) (Axtell and Staskawicz, 2003; Mackey et al., 2003). RIN4 furthermore act in concert with plasma membrane H\(^{+}\)ATPases to regulate stomatal apertures in response to pathogen attacks (Liu et al., 2009). It is therefore possible that RIN4 is targeted by multiple effectors not only to modulate PTI and ETI but also to induce stomatal reopening.

The functional relevance of RIN4 is illustrated by the evolution of at least four structurally unrelated P. syringae effectors that perform three distinct biochemical modifications on RIN4 to regulate host immune functions in order to enhance infection (Axtell and Staskawicz, 2003; Mackey et al., 2003; Mackey et al., 2002; Wilton and Desveaux, 2010). The four known effectors are AvrRpm1, AvrB, AvrRpt2 and HopF2. Further, at least two CNLs, RPM1 and RPS2, constitutively associate with RIN4 and are activated by different effector-mediated alterations (Axtell and Staskawicz, 2003; Mackey et al., 2003; Mackey et al., 2002; Wilton and Desveaux, 2010). Figure 12 illustrates some modifications done to RIN4 by the effectors and the guard involved in defense initiation.

Two RIN4-targeting effectors, AvrB and AvrRpm1, are directed to the host plasma membrane via myristoylation. Both effectors induce phosphorylation of RIN4; though neither are kinases and they are therefore unable to directly phosphorylate RIN4. RIPK (RPM1-INDUCED PROTEIN

<table>
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<tr>
<th>Unchallenged cell</th>
<th>Plant challenged with AvrB or AvrRpm1</th>
<th>Plant challenged with AvrRps2</th>
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<tr>
<td>RIN4</td>
<td>ROC1,RIN4</td>
<td>ROC1,RIN4</td>
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<td>RPM1,RPS2</td>
<td>RIPK,RPM1,RPS2</td>
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<td>Inactive NLR</td>
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Figure 12. In unchallenged cells RPM1 and RPS2 are maintained in an inactive state. Challenge with AvrB or AvrRpm1 results in RIPK-mediated phosphorylation of RIN4 and ROC1-induced conformational changes of RIN4. This is detected by RPM1 which initiates ETI. AvrRps2 is folded by ROC1 and cleaves RIN4 after maturation. This leads to the activation of RPS2 and ETI.
kinase), an Arabidopsis receptor like cytoplasmic kinase, is known to phosphorylate RIN4 at three residues (T21, S160 and T166) (Liu et al., 2011). AvrB physically associates with and enhances RIPK activity by an unknown mechanism, thereby inducing phosphorylation of RIN4 (Chung et al., 2011; Liu et al., 2011) (Figure 12). The phosphorylation of specific RIN4 residues in turn leads to the activation of the NLR RPM1 (Chung et al., 2011; Liu et al., 2011). AvrB and AvrRpm1 are recognized by the same NLR, RPM1, however, the molecular requirements for this recognition are not equivalent and additional interactors differentiate between AvrB and AvrRpm1 induced immunity. One such is TAO1 (target of AvrB operation), a weak TNL that contribute to AvrB-mediated RPM1 activation but has no effect on AvrRpm1 recognition (Eitas et al., 2008). Specifically T166 phosphorylation is induced by AvrB and AvrRpm1 (Chung et al., 2011; Liu et al., 2011). Mimicking RIN4 phosphorylation (T166D) suppresses PTI responses and is sufficient to activate RPM1 in absence of pathogens effectors (Chung et al., 2011; Chung et al., 2014; Liu et al., 2011). Further, RIN4 mutants unable to be phosphorylated at T166 cannot support effector-mediated RPM1 activation (Chung et al., 2011). To further complicate things, the cysteine protease AvrPphB, an effector expressed by P. syringae, was recently found to block the recognition of AvrB by cleaving RIPK and thereby hindering RIN4 phosphorylation (Russell et al., 2015).

The cyclophillin ROC1 (ROTAMASE CYCLOPHILIN 1) also directly interact with, and isomerize RIN4, and has further been implicated in the activation of RPM1 (Li et al., 2014b). AvrB-induced phosphorylation of RIN4 weakens its interaction with ROC1, resulting in conformational change of RIN4, which is then sensed by RPM1 (Li et al., 2014b). ROC1 thus negatively regulates RPM1-induced immunity through its direct interaction with RIN4 and by maintaining RIN4 in an inhibitory conformation (Li et al., 2014b). The role of ROC1 is illustrated by silencing of ROC1 in Arabidopsis, which leads to enhanced resistance to Pst. syringae strains expressing AvrB whereas plants overexpressing ROC1 are compromised in resistance to these (Li et al., 2014b).

ROC1 furthermore plays a role in maturation of the effector AvrRpt2 (Coaker et al., 2006) (Figure 12). The activity of ROC1 is needed for proper folding of AvrRpt2 after its delivery into the plant cell during infection (Coaker et al., 2006). In ROC1 mutants, lacking PPIase enzymatic activity, AvrRpt2 is unable to gain properly folding and activity (Coaker et al., 2006). The requirement of ROC1 for proper folding may reflect a tight association of AvrRpt2 and the host immune system during host-pathogen co-evolution (Li et al., 2014b).

AvrRpt2 cleavage sites are found in RIN4, and cleavage results in disappearance of a larger part of RIN4 leaving only a small C-terminal RIN4 fragment in the membrane (Kim et al., 2005). This has
led to a model where AvrRpt2 cleaves RIN4, releasing it from the membrane, disrupting proper RPM1 localization and accumulation. This cleavage thus blocks the RPM1 recognition of AvrRpm1 or AvrB (Kim et al., 2005). The NLR RPS2 is activated by the degradation of RIN4 following cleavage by AvrRpt2 (Axtell and Staskawicz, 2003). Thus, cleavage of RIN4 by AvrRpt2 removes most of the RIN4 from the membrane, presumably altering interactions with, and regulation of, both RPM1 and RPS2 (Kim et al., 2005). Structure-function analysis suggests that RIN4 keeps RPS2 in an inactive state until it is cleaved. This correlates with rin4 null mutations being lethal in Arabidopsis if RPS2 is functional, illustrating inappropriate activation of RPS2 in the absence of RIN4 (Axtell and Staskawicz, 2003; Mackey et al., 2003).

As described, RIN4 act as a common guardee, targeted by different effectors and at the same time guarded by different NLRs through different molecular modifications. The RIN4 system thus nicely illustrates how plants and pathogens have adopted mechanisms to cope with each other, and how plants have evolved to guard and protect hubs of host proteins with important regulatory roles in immunity, which is targeted by multiple effectors.

**Partnering up – NLR pairs**

Individual NLRs are, in most cases, sufficient to mediate effector recognition and confer resistance. However, in recent years, an increasing number of cases have been reported where two NLRs are required for resistance in both mono- and dicots (Cesari et al., 2013; Eitas and Dangl, 2010; Narusaka et al., 2009; Saucet et al., 2015; Sinapidou et al., 2004) (Figure 13).

The first demonstration of two NLRs working as a pair in plants, was the finding that both the CNLs, RPP2A and RPP2B, are required for resistance to the oomycete *H. arabidopsidis* isolate Cala2, and that mutations in either one caused loss of resistance (Sinapidou et al., 2004). RPP2A/
RPP2B are encoded by adjacent genes and together complement an incomplete resistance conferred by each single gene (Sinapidou et al., 2004).

NLRs working together to mediate recognition and immune signaling have also been found in animals (Eitas and Dangl, 2010). In mice, recognition and response to either bacterial flagellin or the T3SS needle requires ligand-dependent interactions of the NLR NLRC4 with either NAIP5 or NAIP1/NAIP2, respectively (Kofoed and Vance, 2011; Tenthorey et al., 2014). Activated NAIP2 triggers conformational changes in an inactive NLRC4 and NAIP2/NLRC4 can then form a heterodimer. This in turn, activates and successively recruits more NLRC4s to form a large complex in a domino-like reaction (Hu et al., 2015) (Figure 14). Thus, information about animal NLR oligomerization and activation may be helpful for understanding the activation of their plant counterparts. However, it remains to be proven whether or not NLRs in plants also form higher order complexes.

Most known NLR pairs in Arabidopsis are linked, fitting well with NLR gene families often existing in genomic clusters (Meyers et al., 2003; Zhou et al., 2004). The two NLRs are thus often in a head-to-head orientation with a shared promotor (Birker et al., 2009; Narusaka et al., 2009). Furthermore, it is proposed that in pairs, each partner accomplishes only a subset of the functions, frequently executed by one individual non-paired NLR (Cesari et al., 2013). In these paired NLR systems, for the immune complex to activate, an intact P-loop is often only required in one of the partners, but dispensable in the other (Cesari et al., 2014b; Sohn et al., 2014). This supports that each partner have adopted a separate role, one as a sensor and one as the executor and thus have evolved to be functionally specialized (Duxbury et al., 2016). The distribution of sensor, switch and signaling functions between the two NLRs in a pair can only work if a fine-tuned association is maintained through coevolution, analogous to the cooperation between the domains within a single

Figure 14. A schematic diagram for PrgJ-induced assembly of the wheel-like structure of a PrgJ-NAIP2-NLRC4 complex. Binding PrgJ activates NAIP. Activated NAIP recruits and activates a NLRC4 protein. Active NLRC4 recruits and activates more NLRC4 in a domino-like reaction. The completed inflammasome promotes host defense.
NLR (Slootweg et al., 2013; Wang et al., 2015). Thus, close proximity in the genome is advantageous as it lowers the risk of mispairing due to crossover events (Sukarta et al., 2016). Future work elucidating how each member of NLR pairs functions will significantly advance our understanding of plant immune perception and signaling.

Two to tango – RPS4/RSS1

One well-described example of two NLRs working in pair is RPS4 and RRS1. The two NLRs function in concert to mediate recognition of unrelated effectors from at least three different pathogens; the bacterial effector AvrRps4 from \textit{P. syringae}, PopP2 from the bacterium \textit{R. solanacearum} and an unknown effector the fungal pathogen \textit{Colletotrichum higginsianum} (Birker et al., 2009; Narusaka et al., 2009; Sohn et al., 2012; Williams et al., 2014). RPS4 is a classical TNL while RRS1 has an additional C-terminal WRKY motif, characteristic for a group of plant transcription factors (Heidrich et al., 2013). RRS1 is encoded by a gene immediately adjacent to \textit{RPS4}, in ahead-to-head orientation typical of paired \textit{NLRs} (Birker et al., 2009; Narusaka et al., 2009).

RPS4 and RRS1 exist as heterodimers in resting state. This heterologous association is required to form a functional effector recognition complex. Upon effector perception the TIR domain of RRS1 releases the RPS4 TIR domain, allowing it to form a signaling competent homodimer (Williams et al., 2014) (Figure 15). An intact P-loop of RPS4 is required for both AvrRps4- and PopP2-triggered cell death, but that effector-triggered HR was not affected by mutations in the RRS1 P-loop. This supports that RSS1 acts as a sensor, targeted by several effectors, while RPS4 functions as the signaling/executor NLR (Sohn et al., 2014; Williams et al., 2014).

![Figure 15: RPS4 and RRS1 form a hetero-complex in which the signaling NLR (RPS4) is repressed by the sensor NLR (RRS1). Induction of immunity by RPS4/RRS1 involves disruption of TIR domains hetero-interaction and subsequent homo-dimerization of RPS4 TIR domain.](image)

Different alleles of \textit{RRS1} have different specificities. RRS1 in \textit{Arabidopsis} accessions Niederzenz (Nd) and Ws-2 (\textit{RRS1-R}) confers recognition (in concert with RPS4) of PopP2 and AvrRps4 (Tasset et al., 2010; Williams et al., 2014). The Colombia (Col) allele of \textit{RRSI} (\textit{RRS1-S}) confers AvrRps4,
but not PopP2, recognition (Deslandes et al., 2002). RPS4 and RRS1-R (but not RRS1-S) also confers resistance to the fungus \textit{C. higginsianum} (Birker et al., 2009; Narusaka et al., 2009). The main difference between RRS1-R and RRS1-S is 90 additional residues present at the C-terminal of RRS1-R (Le Roux et al., 2015). These residues thus might be required for resistance signaling and may be the cause of the differential immunity between the different \textit{RRS1} alleles. Both PopP2 and AvrRps4 interact with RRS1. While AvrPopP2 interacts specifically with the WRKY domain AvrRps4 has been shown to interact with additional domains (Sarris et al., 2015). PopP2 was shown to acetylate the WRKY domains of both RRS1-S and RRS1-R, disrupting their DNA association (Le Roux et al., 2015; Sarris et al., 2015). Acetylation of RRS1-R abolishes the capacity to recognize AvrRps4. Furthermore, acetyl-lysine mimic substitution alleles of \textit{RRS1-R}, but not \textit{RRS1-S}, trigger effector-independent RPS4 dependent defense activation (Le Roux et al., 2015; Sarris et al., 2015). Both AvrRps4 and PopP2 have been shown to interact with other WRKY transcription factors which also are acetylated by PopP2 (Le Roux et al., 2015; Sarris et al., 2015). The PopP2 acetylation inhibits DNA-binding activities of plant-defensive WRKY transcription factors thereby disabling their transcriptional functions (Le Roux et al., 2015). This is consistent with the hypothesis that RRS1s WRKY domain acts as a decoy, and that the RPS4/RRS1 NLR pair enables plants to detect effectors that interfere with WRKY transcription factor functions (Le Roux et al., 2015; Sarris et al., 2015). It is however still an unsolved puzzle whether or not RRS1 is a true decoy or if it participates as a transcription factor in the transcriptional reprogramming that occurs subsequent to defense initiation (Duxbury et al., 2016).

\textbf{RPS4B/RRS1B}

Interestingly, several close paralogues of \textit{RPS4} and \textit{RRS1} are also linked in a head-to-head orientation in \textit{Arabidopsis} (Narusaka et al., 2009). Recently one of those pairs, RPS4B and RRS1B, was found to mediate resistance to AvrRps4, but not PopP2 (Saucet et al., 2015). Mixed inappropriate pairs (RPS4/RRS1B or RPS4B/RRS1) do exist in some ecotypes, but they are unable to recognize AvrRps4 and PopP2 (Saucet et al., 2015). This suggests that despite the similarity in motif prediction these NLRs have evolved a particular inter-molecular specificity for function and that these proteins therefore must pair with their appropriate respective partner for function (Saucet et al., 2015).
Treasure Your Exceptions – RPM1 and TAO

The functional interaction of one CNL and one TNL has also been described in the case of RPM1 and TAO1 (Eitas et al., 2008). RPM1 and TAO1 were found to act additively to generate a full defense response against *P. syringae* expressing AvrB, but not AvrRpm1 (Eitas et al., 2008). TAO1 is further required for AvrB-induced chlorosis in *rpm1* host plants (Eitas et al., 2008). It is therefore suggested that TAO1 is a weak NLR working in concert with RPM1 for full resistance and mediates AvrB-induced chlorosis. However, RPM1 and TAO1 are not genetically linked, and RPM1 can still recognize AvrB in the absence of TAO1, suggesting that the interaction of these two NLRs is mechanistically different from the other pairs described (Eitas et al., 2008).

Characterization of RPM1-TAO1 has shown that disease resistance can be mediated by NLR pairs of mixed TIR and CC sub-classes (Eitas et al., 2008; Peart et al., 2005). This supports a concept where CNLs and TNLs, which typically trigger different response pathways, can additively contribute to resistance allowing a more complex immune response. This is also the case in tobacco where the CNL NRG1, has been shown to be specifically involved in defense responses mediated by the TNL N (Peart et al., 2005). The characterization of RPM1 and TAO1 as a pair further indicates that the function of one NLR does not always require the partner NLR (Eitas and Dangl, 2010).

**Integrated decoy/sensor domains**

Plant NLR pairs often include of a sensor NLR which possesses an additional domain (Figure 13). Effector binding to this domain facilitates activation of a second signaling NLR; normally with classical domain architecture (Cesari et al., 2014a; Kroj et al., 2016) (Figure 13). These extraneous domains of the sensor have, in some cases, been suggested to resemble the primary effector targets in the host and are subsequently thought of as decoys that have been integrated into an NLR (Cesari et al., 2014a; Kroj et al., 2016; Wang et al., 2015). The additional domains have in most cases evolved by duplication of an effector target followed by fusion into the NLR. The NLR-integrated domain mimics effector binding/substrate property of the original effector target to enable pathogen detection (Cesari et al., 2013; Le Roux et al., 2015; Sarris and Jones, 2015) (Figure 16).
NLR fusions with additional domains have been found in both mosses and across all lineages of flowering plants (Sarris et al., 2016). Sarris et al. reported that on average 10% of plant NLRs contain additional integrated domains (Sarris et al., 2016). Since these integrated domains are found across most plant species, it is likely that this integrated decoy/sensor recognition is an evolutionarily conserved mechanism of diversification in flowering plants (Cesari et al., 2014a). This theory is supported by a large overlap between fusion domains and effector targets (Sarris et al., 2016).

Recently, Wu et al. proposed an expansion for the integrated decoy model, by suggesting that NLR-integrated decoy domains do not necessarily have to be defective mimics (Wu et al., 2015). They might still carry their biochemical activity, continuing to function in the effector targeted pathway even as an extraneous domain within a classic NLR architecture (Wu et al., 2015). This is not yet documented but is supported by the comparative analysis done by Sarris et al. They found that kinases fused to NLRs in various plant species predominantly encode full-length kinase domains that are potentially catalytically active (Sarris et al., 2016). Furthermore, there is a clear prevalence of domains involved in protein activities associated with protein kinases, DNA-binding domains and protein-protein interactions found in fusion with NLRs across several plant species (Kroj et al., 2016; Sarris et al., 2016). This supports the hypothesis that these integrated domains often serve as baits for pathogens, and that the same pathways are targeted by pathogens across multiple plant species. Several cases of fusion between NLRs and known guardes were found. Among these, NLR-RIN4 fusions were found in numerous species, including barley and rice (Sarris et al., 2016).

So far, all well-studied NLRs with integrated domains functions in pair with a NLR partner, which have classical NLR domain architecture (Figure 17). This could possibly be due to integration of a non-canonical sequence being incompatible with some NLR functions. The second member of the pair is therefore required to complement the compromised functions (Ellis, 2016) (Figure 17).
is supported by data from studies of pairs with integrated domains, where that one NLR from the pair acts as a sensor and the second as the trigger of defense activation. Duxbury et al. have suggested that when multiple NLRs evolve to function as one unit, redundancy arises in duplicated domains (Duxbury et al., 2016). This will lead to relaxed evolutionary constrains on individual NLR domains and may provide the necessary flexibility for the integration of extra domains (Duxbury et al., 2016). The separation of functions among the partners in an NLR pair may help prevent inappropriate immune activation, which is costly and often comes at the expense of plant growth and development.

**With a little help from my friends – Helper NLRs**

In mammals one executor NLR working in concert with multiple sensor NLRs, to broaden specificity, have been reported (Duxbury et al., 2016; Kofoed and Vance, 2011; Tenthorey et al., 2014). A similar signaling role of a single NLR in relaying pathogen recognition via multiple NLRs may also occur in plants. Members of the small ADR1 (ACTIVATED DISEASE RESISTANCE 1) family of CNLs in Arabidopsis (ADR1, ADR1-LIKE 1, and ADR1-LIKE 2) function downstream of other NLRs, namely RPS2, RPP2, and RPP4 (Bonardi et al., 2011). Thus, an emerging model is that NLRs may recruit so-called helper NLRs to form a final signalosome. Helper NLRs are required for the functions of other NLRs and may be involved in relaying the signal downstream of the respective sensor NLRs (Bonardi et al., 2011). Helper NLRs provide a non-canonical route to resistance, for instance by complementing sensor NLRs that may not possess apparent signaling domains (Sukarta et al., 2016). Also, helper NLRs may link signaling between non-nuclear receptors and transcriptional reprogramming.

Know helper NLRs are characterized by an atypical CC domain lacking an EDVID motif, which appears to mediate the intramolecular interactions in classical NLRs (Collier et al., 2011; Rairdan and Moffett, 2006). Helper NLRs appear to be present in a basal clade with two distinct subgroups (Collier et al., 2011; Collier and Moffett, 2009). One subgroup is exemplified by ADR1. The
second subgroup is exemplified by tobacco ADR1 homolog NGR1, which is required for N-mediated immune response, and was the first helper NLR reported (Peart et al., 2005). It is generally seen that helper NLRs transduce signaling of multiple immune receptors and thus cooperate synergistically in various NLR signal transduction routes. The activity of helper NLRs is often demonstrated as being P-loop independent, further discriminating their role from the classical molecular switch NLRs (Sukarta et al., 2016).

ADR1 does not rely on an intact P-loop for function suggesting that ATP binding is not required for signaling. Interestingly Roberts et al found that ADR1-L2 autoactive mutants display a P-loop dependent autoimmune phenotype (Roberts et al., 2013). This indicates that apart from P-loop independent helper activity, ADR1-L2 also exhibits canonical P-loop dependent NLR activity (Roberts et al., 2013). Thus some helper NLRs like ADR1-L2 may be dual functional, illustrating again the complexity of immune signaling in plants. Members of the helper NLR ADR1 family are required for both PTI and ETI mediated by a distinct set of NLRs, which further are dependent on SA signaling for full immune response (Bonardi et al., 2011). ADRs are thus clearly functionally distinct from regular switch NLRs. The ADR1 family is exceptionally conserved among various plant species. Due to the high degree of conservation this family may represent a conserved and potentially ancestral function (Jacob et al., 2013). Furthermore, the involvement of helper NLRs in both PTI and ETI suggests they may act as molecular bridges between the two layers of plant immunity (Sukarta et al., 2016).

**Delivering orders – NLR signaling**

While NLRs play a central role in the activation of resistance following pathogen perception, NLRs alone are not sufficient for the initiation of resistance. Despite the importance of NLR induced immunity in plants and the extensive research in the field for the past few decades, the immediate targets of activated NLRs remain largely unknown. One possible reason for the paucity of identified ETI signaling components could be a very short signaling pathway. Consistent with this, several NLRs localize in the nucleus, and some directly binds transcription factors leading to changes in defense gene expression (Zhu et al., 2010b). However, forward genetic screens have identified some robust ETI signaling components, including downstream targets, ancillary proteins, and chaperones serving as co-activators of resistance (Belkadir et al., 2004b; Hubert et al., 2003; Muskett et al., 2002; Tornero et al., 2002; Aarts et al., 1998).
The presence of a CC or TIR domain in the N-terminus of an NLR dictates the downstream signaling pathways initiated by the NLR (Aarts et al., 1998). CNLs generally require the GPI anchored plasma membrane localized protein NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE 1) for signaling (Century et al., 1995). Multiple TNLs, on the other hand, have been shown to require EDS1 (ENHANCED DISEASE SUCCEPIBILITY 1), PAD4 (PHYTOALEXIN DEFICIENT 4), and SAG101 (SENSCESSION-ASSOCIATED GENE 101) for signaling (Feys et al., 2005; Wagner et al., 2013). The processes by which plant NLRs induce ETI are still poorly understood and despite the clear importance of EDS1/PAD4 and NDR1 neither their biochemical function nor their exact role in immunity is known. Furthermore, how NLRs with diverse localizations trigger a similar set of ETI responses is still a major unanswered question.

The relationship between the CNLs and NDR1 are illustrated by ndr1 mutants, in which the resistance conferred by the CNLs RPS2, RPM1, and RPS5 is compromised (Century et al., 1995; Aarts et al., 1998). In the same way, the eds1 mutant is immune-compromised in P. syringae AvrRps4 resistance which is conferred by the TNLs RPS4/RRS1 and RPS4B/RRS1B (Falk et al., 1999; Saucet et al., 2015; Aarts et al., 1998). Furthermore, it is known that HR cell death triggered by TNLs via EDS1 requires autophagy, whereas HR initiated by CNLs via NDR1 is either autophagy-independent and engages other unidentified cell death mediators (Hofius et al., 2009). Thus, EDS1 and NDR1 operate preferentially in distinct resistance signaling pathways.

However, there are exceptions to these rules and some CNLs, like RPP7, initiate resistance via a pathway(s) that is independent of NDR1 and EDS1 (McDowell et al., 2000; Aarts et al., 1998). While there appears to be conservation in terms of specificity, function, and activation of signaling through both EDS1 and NDR1 in terms of NLR structure, the full mechanism(s) associated with these pathways is unknown. That some NLRs, like RPP7, seem to act in an EDS1 and NDR1 independent way indicates that a third signaling pathway exists in addition to EDS1 and NDR1 (McDowell et al., 2000). It is further proposed that NLRs may mediate immune responses via multiple signaling pathways, rendering it difficult for pathogens to evolve a single effector which simultaneously hampers these multiple signaling pathways (Maekawa et al., 2011). It can thus be speculated that such multiple immune targets downstream of a single NLR could contribute to the robustness against rapidly evolving pathogenic effectors (Jacob et al., 2013). This would also explain the conservation of plant NLR signaling mechanisms across plant species, illustrated by the Arabidopsis NLR pair RPS4/RRS1, which also confers resistance when expressed in cucumber (Cucurbitaceae), N. benthamiana, and tomato (Narusaka et al., 2013).
Of the primary signaling components required for the activation of NLR-mediated resistance, the role of EDS1 in defense signaling is best understood. *Arabidopsis* EDS1 interacts directly with the two sequence-related signaling partners, PAD4 and SAG101 (Feys et al., 2001; Feys et al., 2005) (Figure 18). EDS1, which is present in the cytoplasm and nucleus, is detected preferentially in the nucleus in the presence of SAG101. The presence of PAD4 restores the cytoplasmic localization of EDS1. This suggests that coordination between different EDS1-PAD4 and EDS1-SAG101 complexes might underlie immunity regulation (Feys et al., 2005; Rietz et al., 2011). However, a ternary SAG101-EDS1-PAD4 nuclear complex has been reported, representing another potential signaling-active form (Zhu et al., 2011). The SAG101-EDS1-PAD4 ternary complex, which is detected primarily in the nucleus, is redirected to cytoplasm in the presence of extranuclear EDS1 (Figure 18). This indicates that the protein localization changes in relation to the subcellular localization and/or relative levels of their interacting partners (Zhu et al., 2011).

EDS1 interact with the *Arabidopsis* NLRs RPS4, RPS6 and SNC1 and it is a possibility that these interactions may be required to produce a mobile NLR signal necessary for defense initiation (Bhattacharjee et al., 2011; Heidrich et al., 2011).

**Changing the program – NLRs and transcriptional reprogramming**

As a more direct regulatory function, several NLRs are known to directly recruit and associate with components involved in transcriptional reprogramming. This includes interaction with transcription factors, or activation of MPK cascades upon effector perception (Bhattacharjee et al., 2011; Mukhtar et al., 2011). The barley NLR MLA10 binds two WRKY transcription factors, WRKY1 and WRKY2, in an effector-dependent manner (Shen et al., 2007). The WRKYs act as repressors of PTI by inhibiting the activity of MYB6, a positive regulator of PTI and MLA10-induced resistance (Chang et al., 2013). MYB transcription factors have numerous roles in signaling and development,
and are involved in signaling connected to plant hormones important for stress and defense responses namely SA and JA. MLA10 also binds MYB6 and this interaction is needed to release MYB6 from being antagonized by the WRKYs (Chang et al., 2013). MLA10 activation thus allows rapid transcriptional reprogramming in response to pathogen perception by interfering with the WRKY1/2 repressor function on MYB6 (Figure 19). This direct targeting of PAMP-activated WRKY repressors by MLA10 represents a molecular shortcut independent of the classical NLR signaling pathways (Deslandes and Rivas, 2011).

Both RPS4 and the SNC1 have been found to interact with the transcriptional activators. SNC1 associates with the transcriptional co-repressor TPR1 (Zhu et al., 2010b). The SNC1 TPR1 interaction induces immunity by negatively regulating the expression of the defense suppressors DND1 and DND2 (Zhu et al., 2010b). Nuclear-localized NLRs mediate transcriptional reprogramming via interaction with transcription factors across species (Jacob et al., 2013). However, considering the massive immune response triggered by activation of NLRs, all ETI reprogramming cannot be explained by direct NLR regulation of transcription factors (Spoel and Dong, 2012). Furthermore, several NLRs are known not to be present in the nucleus and are not interacting with known transcription factors.

Both MAPK cascades and CDPKs have been found to be fundamental components of ETI, and activation of these kinases may explain how plasma membrane-associated NLRs transmit signaling to the nucleus in order to induce transcriptional reprogramming (Meng and Zhang, 2013) (Figure 20). The activated plasma membrane CNLs, RPS2 and RPM1, trigger MPK3 and MPK6 phosphorylation, which in turn drives transcriptional reprogramming (Tsuda et al., 2013). MPK3 and MPK6 are functionally redundant and both interact with and phosphorylate the WRKY transcription factor WRKY33 to regulate transcription.

**Figure 19.** MLA10 directly link effector perception with transcriptional regulation. In unchallenged cells WRKY1/2 works as transcriptional repressors. MLA10, which depends on the chaperone complex for proper folding is found to cycle between nucleus and cytoplasm. Upon effector perception the activated MLA10 bind to WRKY1/2 and thereby release MYB6. Further MLA10 bind free MYB6 to initiate defense gene expression.
Also RPS4 induced immunity involves MPK cascades and interestingly, it has been found that ETI triggered by nuclear RPS4 involves significantly lower MAPK activation compared to that triggered by RPS4 in the cytoplasm (Tsuda et al., 2013). So far there are limited data elucidating the role of CDPKs in regulation of gene expression during ETI. However a group of CDPKs was recently found to act as signaling mediators between the NLRs and WRKYs (Gao et al., 2013). Thus both MAPKs and CDPKs seem to provide important molecular links between certain NLRs and transcriptional reprogramming.

Additionally, as MPK4 has been connected to PAT1 and mRNA decapping, it is obvious to speculate that the massive transcriptional changes upon defense initiations may in part be regulated by altering mRNA decay rates as well (Roux et al., 2015). Already mRNA decay regulation by MPKs has been implicated in stress responses in both plants and other organisms, although it remains elusive how this affect RNA turnover and transcriptional changes (Roux et al., 2015).

**The hormonal side of immunity**

Phytohormones like SA, JA, and ET, play key roles in signaling NLR-mediated defense (Leon-Reyes et al., 2010; Lu, 2009; Tsuda et al., 2008). In SA-mediated responses NPR1 act as a transcription co-factor inducing defense through regulation of WRKYs. Increased SA levels result in formation of monomers of NPR1, this in turn lead to NPR1 re-localization to the nucleus where it interacts with transcription factors resulting in changes in defense gene expression (Lindermayr et al., 2010). In *Arabidopsis* and other higher plants, local and systemic defense responses are controlled by the balanced action of distinct, but partially interconnected pathways involving SA, JA, and ET (Bostock, 2005; Leon-Reyes et al., 2010). SA seems to antagonize JA-mediated responses and vice versa. ET fine-tunes appropriate defense responses by inhibiting SA-mediated suppression of JA defense responses (Leon-Reyes et al., 2010) (Figure 21). This correlates with a general perception that, resistance to biotrophs requires SA-dependent signaling pathways, whereas...
resistance to necrotrophs relies on JA/ET-dependent pathways (Glazebrook, 2005) (Figure 21). The combined actions of SA and JA thus play a role in modulating appropriate defense responses dependent on the class of pathogen.

As for many other key parts of defense, mutations disrupting SA signaling result in compromised defense, whereas overexpression of SA results in enhanced resistance (Lu, 2009). Thus, mutants such as npr1, which are impaired in SA responsiveness (Cao et al., 1997), or mutants defective in pathogen-induced SA accumulation, such as eds1 and pad4, exhibit enhanced susceptibility to pathogen infection and show impaired defense gene expression (Cao et al., 1997; Falk et al., 1999).

**The art of being in the right place at the right time – NLR localization and mobility**

Plant NLRs have diverse subcellular localizations, including targeting to the endoplasmic reticulum, plasma membrane, nucleus and cytosol (Caplan et al., 2008a; Shen et al., 2007). Of the ~150 Arabidopsis NLRs 80% have been predicted to be nuclear, while only 8% were predicted to be cytoplasmic and 8% found in the chloroplasts (Caplan et al., 2008a). It is likely that plant NLRs is targeted to diverse sub-cellular locations depending on where their cognate effectors are present (Qi and Innes, 2013). Reports indicate, that some NLRs re-localize following effector detection, and such re-localization may reflect differences in signaling pathways dependent on the localization (Qi and Innes, 2013) (Figure 22). The possibility of compartment specific activities of NLRs supports, that a single NLR may interact with multiple structurally distinct downstream components to initiate immune responses. Dynamic nuclear re-localization of NLRs after effector recognition, suggests that these NLRs may directly regulate transcription factors to induce defense signaling or even function as transcription factors themselves (Shen et al., 2007; Tameling and Baulcombe, 2007; Wirthmueller et al., 2007). Consistent with this, several plant NLRs have been shown to
accumulate in the nucleus upon effector-induced activation (Caplan et al., 2008a; Cheng et al., 2009; Shen et al., 2007; Wirthmueller et al., 2007).

Coordinated nucleo-cytoplasmic trafficking of plant NLRs is in some cases required for the full activation of defense responses (Figure 22). This suggests that a single NLR may activate distinct signaling pathways in the cytoplasm and nucleus. RPS4 are found in both cytoplasm and nucleus (Wirthmueller et al., 2007). The C-terminal extension domain of RPS4 is predicted to have a putative bipartite nuclear localization signal, and RPS4 nuclear localization is dependent on the C-terminal domain (Wirthmueller et al., 2007). RPS4 confers resistance to \textit{P. syringae} strains expressing the effector AvrRps4. Forced nuclear accumulation of AvrRps4 is sufficient to activate RPS4-mediated bacterial growth inhibition, but blocks RPS4-mediated HR (Heidrich et al., 2011). Sequestration of AvrRps4 in the cytosol using a nuclear export signal significantly impairs RPS4-mediated resistance but only moderately reduces RPS4-mediated HR (Heidrich et al., 2011; Qi and Innes, 2013). This indicates that RPS4 defense responses are dependent on a nuclear re-localization of the effector, but the activation mechanism for HR induction is independent of this re-localization.

MLA10 also shows nucleo-cytoplasmic partitioning with distinct location-specific activities (Bai et al., 2012; Shen et al., 2007). In unchallenged cells the majority of MLA is located in the cytoplasm with only a fraction found in the nucleus. However, upon infection the nuclear pool increases significantly (Shen et al., 2007). Interestingly, MLA10 does not contain a known nuclear localization signal (Shen et al., 2007) and is therefore suggested to be “piggy-back” transported into the nucleus. When expressed in the nucleus, MLA10 confers disease resistance, whereas cytoplasmic MLA10 is sufficient and required for HR (Bai et al., 2012). Nuclear exclusion abolishes MLA10-mediated resistance. In general, nucleo-cytoplasmic partitioning of plant NLR proteins may prove to be a regulatory mechanism for differential activation of downstream signaling.

NLRs are in general large in size, and therefore too big to simply diffuse though nuclear pores, they must therefore be actively transported into the nucleus (Liu and Coaker, 2008). Consistent with this, plants carrying mutations in genes encoding components of the nucleocytoplasmic transport machinery, often display defects in resistance to pathogens (Cheng et al., 2009; Palma et al., 2005). The TNL SNC1 possesses a canonical NLR structure and further contains both a nuclear localization signal and two nuclear export signals (Cheng et al., 2009). Mutations in \textit{SNC1} result in autoimmunity. The importance of nuclear import was established by the identification three MOS proteins (MODIFIER OF SNC1) involved in nuclear transport. Mutations in these suppress \textit{snc1}
autoimmunity (Cheng et al., 2009; Palma et al., 2005). Mutations in MOS3, part of the putative plant Nup107-160 nuclear pore subcomplex, suppress the constitutive autoimmune phenotypes of sncl mutants and further suppress resistance mediated by the NLRs RPP4, RPM1 and RPS4. Also mos6 mutants partially suppress sncl autoimmune phenotypes and the mos6 single mutant shows enhanced disease resistance to H. arabidopsidis (Palma et al., 2005).

MOS7 involved in general nuclear transport and are required for PTI, SAR and NLR-mediated resistance. Complete loss-of-function mos7 mutants are lethal (Cheng et al., 2009). Interestingly, partial loss of MOS7 impairs the nuclear accumulation of the defense regulators NPR1, EDS1 and SNC1, but does not affect the nuclear distribution of non-defense related proteins, suggesting MOS7 preferentially import immune-related proteins (Cheng et al., 2009).

Several NLRs have been found to contain nuclear localization signals. However, multiple examples of NLRs lacking these classical signals have been reported to depend on cytoplasmic to nucleus re-localization, like MLA10 (Shen et al., 2007; Tameling and Baulcombe, 2007). These NLRs may interact with carrier proteins, or they could possess nuclear localization signals that have not yet been identified. These NLRs might also be imported by still unknown nuclear localization signal-independent pathways (Liu and Coaker, 2008). Studies have shown that 43% of known nuclear-localized proteins in yeast do not possess a detectable nuclear localization signals (Lange et al., 2007), supporting a hypothesis that multiple NLRs are re-localized despite lack of known mechanisms.

Not all NLRs are re-localized upon effector-induced activation. The Arabidopsis CNLs RPS5 and RPM1 are found at the plasma membrane, and have been shown to require this localization to function (Qi et al., 2012). This requirement likely reflects the localization of the cognate pathogen
effector proteins. RPS5 detects the effector AvrPphB, which is a cysteine protease expressed by *P. syringae* (Ade et al., 2007).

Taken together these reports highlight the importance of appropriate subcellular distribution and extensive coordination across subcellular compartments for full resistance.

**Keeping the troops in line – Regulating of the NLRs**

Control of NLR homeostasis is crucial for NLR function and for plant survival, as NLR over-accumulation may lead to increased immune signaling and autoimmune phenotypes in non-infected plants (Li et al., 2001). Conversely, plants with loss-of-function NLRs are often more susceptible to specific pathogen attacks. To prevent inappropriate activation, NLRs exist in an inactive state that relies on negative regulation exerted by the LRR on the NB-ARC domain (Ade et al., 2007; Takken and Tameling, 2009). Increasing evidence additionally suggests that regulation of plant NLR homeostasis occurs at many different levels, including transcription, RNA processing, and protein folding and degradation (Li et al., 2015). Furthermore, for NLRs known to monitor the presence of an effector target, the presence this host protein is often required for the accumulation of the NLR, as is the case for RPM1 and RIN4 (Belkhadir et al., 2004a). In absence of RIN4, RPM1 does not accumulate (Belkhadir et al., 2004a).

NLRs require molecular chaperones to facilitate their folding, stability and conformational changes (Hubert et al., 2003; Muskett et al., 2002; Shirasu, 2009). In line with this, plant NLRs are substrates of a structurally and functionally conserved chaperone complex that consists of HSP90 (HEAT SHOCK PROTEIN 90) and its co-chaperone SGT1 (SUPPRESSOR OF THE G2 ALLELE OF SKP1) (Li et al., 2015; Shirasu, 2009). Furthermore, RAR1 (REQUIRED FOR MLA12 RESISTANCE 1) has been found to regulate the HSP90-SGT1 complex, resulting in further stabilization of NLRs (Shirasu, 2009). The chaperone complex maintain NLRs in a recognition-competent state and, after effector recognition, it facilitates the conformational changes of the NLR needed for downstream signaling (Spoel and Dong, 2012). Mutations in these chaperones result in a lower accumulation of a number of NLRs, and thus affect the immune responses. This suggests that chaperone-assisted folding is critical for the stability and potentially the formation of immune receptor complexes (Kadota and Shirasu, 2012; Shirasu, 2009). This is supported by several lines of evidence: RPM1 levels are reduced in *Arabidopsis rar1* and *hsp90* mutants, and the amount of RPS2 is reduced in *Arabidopsis rar1* (Belkhadir et al., 2004b; Hubert et al., 2003; Torner et al., 2002). Additionally, silencing of *SGT1* resultes in low levels of N soluble protein, which is
consistent with SGT1 being involved in the elicitor-induced stabilization of N (Mestre and Baulcombe, 2006).

NLR turnover is also important to avoid over-accumulation of NLRs and the accompanying autoimmunity. In this, the ubiquitin proteasome system plays a major role (Cheng et al., 2011; Huang et al., 2014). Proteins are targeted for degradation by the 26S proteasome through ubiquitination. For this process the activities of the E1 ubiquitin activating enzyme, the E2 ubiquitin conjugating enzyme, and the E3 ubiquitin ligase are all essential (Cheng et al., 2011). Loss of the ubiquitin proteasome system leads to increased accumulation of NLRs and this in turn results in autoimmune phenotypes (Cheng et al., 2011). Levels of both SNC1 and RPS2 NLRs in Arabidopsis have been shown to be regulated by an E3 ligase complex (Huang et al., 2014). snc1 mutants that accumulate the TNL SNC1 exhibit classical autoimmune characteristics, including dwarfism, curled-leaves and enhanced disease resistance (Li et al., 2001). Overexpression of the ubiquitin E3 ligase, which targets SNC1, reduces SNC1 levels and suppresses the snc1 phenotypes. Mutants lacking the E3 ligase have increased SNC1 levels and display autoimmune phenotypes (Cheng et al., 2011). Control of NLR turnover by the ubiquitin proteasome system has so far only been reported for a small number of plant NLRs, but it is likely that many NLRs are degraded via this system. Since malfunctions lead to massive accumulation of NLRs, these mutants may in general be overlooked due to lethality (Li et al., 2015).

Transcriptome analyses of NLR-encoding genes in Arabidopsis have shown that most NLRs are expressed at low levels and exhibit both tissue-specificity and vast ecotype variation (Tan et al., 2007). Furthermore, a number of studies have shown that transcription of NLRs is dynamically regulated by DNA methylation and histone modifications. Both LAZ5 and RPM1 expression were found to be dependent on the histone lysine methyltransferase SDG8 (SET DOMAIN GROUP 8), also known as LAZ2 (Palma et al., 2010). SDG8 are additionally required for full resistance to virulent pathogens and resistance to avirulent pathogens mediated by various NLRs including RPM1 (Palma et al., 2010). Another example is MOS1, which was found to regulate SNC1 expression at the chromatin level. Mutations in mos1 result in altered DNA methylation in a region upstream of SNC1 and thus reduced SNC1 expression (Li et al., 2010b; Li et al., 2011). Expression of another NLR, RPP4 which is encoded in the same cluster as SNC1, is also altered in mos1 mutants (Johnson et al., 2016; Xia et al., 2013). These studies emphasize the importance of epigenetic regulation in NLR gene transcription.
In addition to the constitutive and static cellular regulation, NLRs, and thus their activities, seem to follow a dynamic expression and accumulation pattern (Cheng et al., 2013; Wang et al., 2011). This is nicely illustrated by analysis of the expression pattern of *Arabidopsis RPS4*. RPS4, that confers resistance to *H. arabidopsidis*, was shown to be expressed in a rhythmic pattern dependent on the circadian clock (Wang et al., 2011). Interestingly the expression of *RPS4* peaks in the morning, correlating with the time of *H. arabidopsidis* sporulation (Wang et al., 2011). Further it was recently reported that ambient temperature changes lead to pronounced shifts of immune responses (Cheng et al., 2013). Plants preferentially activate ETI signaling at relatively low temperatures (10~23°C), whereas they switch to PTI signaling at moderately elevated temperatures (23~32°C) (Cheng et al., 2013). This correlates with pathogen effector secretion being favored at low temperatures whereas high temperatures lead to bacterial multiplication (Cheng et al., 2013). It is therefore also not surprising that several autoimmune mutants, which can be suppressed by loss-of-function of key ETI components, can also be suppressed by high temperatures (Bruggeman et al., 2015). It seems that plants can regulate expression of specific defense components according to anticipated needs providing yet another layer of NLR control.

Taken together, this stresses the importance of proper NLR regulation in order to avoid inappropriate NLR activation and thus autoimmunity, or unnecessary NLR-induced immunity resulting in enhanced infection and damage.

**Self-destructive plants – Autoimmune mutants**

Tight control of plant immunity is needed to avoid activation of defense responses in the absence of pathogens, as failure to do so can lead to autoimmunity which compromises plant growth and development. In the same way erroneous activation of programmed cell death responses is detrimental to the plant and is therefore also under tight regulation.

Autoimmunity in general refers to activation of processes that are harmful to the organism, such as inflammation/immune responses, in the absence of an appropriate trigger. In humans, defects in innate immune receptors such as TLRs and NLRs or self-recognition by lymphocytes and antibodies in the adaptive immune system can lead to autoimmune diseases (Kawai and Akira, 2009).

Similarly, autoimmunity can occur in plants and a number of forward genetic screens have identified many autoimmune mutants, in maize, rice, barley, and *Arabidopsis* (Bruggeman et al., 2015; Lorrain et al., 2003; Moeder and Yoshioka, 2008). The causal mutations are often found to be
loss-of-function or gain-of-function variants of NLRs themselves, key regulators of immunity or cell death, and components of defense hormone synthesis and signaling (Bruggeman et al., 2015). Also uncontrolled ROS production, disrupted Ca\(^{2+}\) signaling, and failed MAPK signaling may be the underlying causes of autoimmune phenotypes (Bruggeman et al., 2015).

Plant autoimmune mutants are associated with dwarfism and enhanced resistance. These mutants typically show additional common phenotypes, including elevated expression of defense genes such as \textit{PRI} and \textit{PR2}, increased SA levels, increased ROS levels, stunted growth and necrotic or chlorotic lesions (Lorrain et al., 2003; Moeder and Yoshioka, 2008). Since many such mutants display microscopic cell death at least under some environmental conditions, resembling HR, they are often referred to as lesion mimic mutants as well.

Defects in calcium signaling may also lead to autoimmunity in plants. An example of this is seen in the \textit{dnd1} and \textit{dnd2} autoimmune mutants (Clough et al., 2000; Jurkowski et al., 2004). The \textit{dnd} null mutants exhibit autoimmune phenotypes, including constitutive expression of \textit{PR} genes, elevated accumulation of SA, and enhanced resistance to a broad spectrum of pathogens (Clough et al., 2000). \textit{dnd} mutants are however impaired in mounting HR. DND1 and 2 are members of the cyclic nucleotide-gated ion channel family and play important roles in regulating cytosolic calcium influx (Clough et al., 2000). It is suggested that the inability of the \textit{dnd} mutants to change the cytosolic level of calcium renders them unable to induce HR illustrating the importance for calcium signaling in this process. However, since \textit{dnd} mutants retain resistance, calcium signaling seems dispensable to some extent (Jurkowski et al., 2004).

Due to the classic cell death phenotype, it has often been assumed that the autoimmunity-causing deficiencies would be found in genes encoding proteins involved in either programmed cell death or negative regulators of immunity. However, recently it has become clear that inappropriate activation of NLRs is significantly associated with autoimmunity (Bruggeman et al., 2015; Rodriguez et al., 2016; Roux et al., 2015; Zhang et al., 2012). A recent report from Bruggeman \textit{et al.} showed that 40\% of the listed 49 lesion mimic mutants are suppressed by loss-of-function mutations in key components of immunity (Bruggeman et al., 2015; Rodriguez et al., 2016). This includes mutations in EDS1, PAD4 and interference with SA, either by mutations in SID2, an isochorismate synthase required for SA biosynthesis or by expression of the bacterial salicylate hydroxylase NahG which degrades SA (Bruggeman et al., 2015).

Additionally, a number of \textit{Arabidopsis} autoimmune mutants are caused by gain-of-function mutations in NLRs. One example of this is \textit{snc1} (Li et al., 2001; Zhang et al., 2003). A single amino
acid change in the linker region between the NB and LRR domains leads to constitutively activation of the SNC1 protein (Zhang et al., 2003). snc1 gain-of-function mutants exhibit autoimmune phenotypes, but interestingly do not have spontaneous cell death (Zhang et al., 2003). Over-accumulation of SNC1 also results in autoimmunity, as seen in CRP1 loss-of-function mutants (Gou and Hua, 2012). CRP1 regulates levels of SNC1 and loss of CRP1 leads to elevated levels of SNC1 and autoimmunity (Gou and Hua, 2012). Also plants with mutations in E3 ligases show SNC1-dependent autoimmunity due to accumulation of the NLR (Cheng et al., 2011). As mentioned before, the snc1 phenotypes can be suppressed to different extent by a set of mos mutants (Cheng et al., 2009; Palma et al., 2005) (Figure 23).

Figure 23. Morphology of snc1, snc1/mos3, snc1/mos6 and wild type plants. snc1 autoimmunity is dependent on the nuclear pore complex and is suppressed by mutations in MOS3 or MOS6. (Zhu et al., 2010a)

Characterizations of these MOS genes have been instrumental in understanding the fine tuning of plant defense responses and have shown that SNC1-mediated resistance is regulated by many processes and pathways (Gou and Hua, 2012). This includes proper nucleo-cytoplasmic transport, turnover and chromatin modification. The involvement of NLRs in autoimmunity is often deduced from NLR mutations suppressing or enhancing the autoimmune phenotypes. Several examples of this are known from Arabidopsis including suppression of acd11 by mutations in the NLR LAZ5 (Palma et al., 2010).

Other evidence linking autoimmunity and NLRs may be hybrid necrosis, a syndrome that is characterized by activation of the immune system due to inappropriate self-recognition in the progeny of crosses among natural accessions (Bomblies and Weigel, 2007; Spoel and Dong, 2012). This concept is illustrated in Figure 23. Large-scale studies on hybrids between natural accessions of Arabidopsis have shown that approximately 2% of the intraspecific hybrids suffer from autoimmunity. This include spontaneous cell death, tissue collapse, leaf lesions, chlorosis, dwarfism, in extreme cases leading to sterility and even death (Bomblies and Weigel, 2007; Chae et al., 2014).
Incompatibility loci often map to rapidly evolving NLR genes or gene clusters, and the phenotypes of these hybrids can typically be suppressed at higher growth temperatures (Bomblies and Weigel, 2007). Most incompatible hybrids develop necrotic spots only at temperatures below 20°C or lower (Bomblies and Weigel, 2007; Chae et al., 2014). This temperature dependent phenotype could indicate that ETI activation is involved. Hybrid necrosis may thus be due to activation of NLR dependent defense responses due to a failure in guard–guardee interactions as illustrated in Figure 24.

NLR dependent autoimmunity can be caused by mutations in guardees (Palma et al., 2010; Roux et al., 2015; Zhang et al., 2012). Knockout mutations that eliminate host guardees mimic the effects of pathogen effectors, and have been found to exhibit NLR mediated cell death (Rodriguez et al., 2016; Roux et al., 2015; Zhang et al., 2012). It is thus possible, that more autoimmune mutants may correspond to gene functions that are guarded by NLRs. A classic example of autoimmunity caused by mutations in a guardee is rin4. RIN4 is guarded by several NLRs including RPS2 as described above (Axtell and Staskawicz, 2003; Mackey et al., 2003). In rin4 RPS2 activates defense in absence of pathogens causing the autoimmune phenotypes (Mackey et al., 2003). rin4 lines expressing RPS2 have been found to be seedling lethal like many other autoimmune mutants (Belkhadir et al., 2004a; Mackey et al., 2003). Embryo/seedling lethality is a challenge when working with these autoimmune mutants, however mutants may also survive to later developmental stages and some even set seeds.

Suicidal plants – acd11 and LAZ5

The acd11 autoimmune phenotypes and runaway cell death have thus been shown to rely on the NLR LAZ5 (Palma et al., 2010). The lethal, recessive acd11 mutant shows autoimmune cell death and constitutive activation of defense responses due to disruption of a ceramide-1-phosphate transfer protein (Brodersen et al., 2002; Simanshu et al., 2014). acd11 autoimmunity is initiated at
an early seedling stage and was shown to be dependent on SA and EDS1-dependent signaling pathways. Autoimmunity in *acd11* can be suppressed by expression of bacterial salicylate hydroxylase NahG or by mutations in *PAD4* or *EDS1* (Brodersen et al., 2005; Feys et al., 2001). *acd11* phenotypic dependency on LAZ5 was identified in a screen for suppressors of the autoimmune phenotype (Palma et al., 2010). It was found that *acd11* autoimmunity requires the histone lysine methyltransferase LAZ2, as well as the TNL LAZ5, whose expression is dependent on LAZ2 activity (Palma et al., 2010). It has additionally been shown that a dominant negative P-loop mutation in LAZ5 completely suppress all *acd11* phenotypes (Palma et al., 2010) (Figure 25).

Interestingly, *laz5* T-DNA insertion mutants show only partial suppression. This suggests that activation of other NLRs may contribute to the *acd11* phenotype (Palma et al., 2010). Suppression of *acd11* phenotypes is however obtained in *acd11/laz2* double mutants. This is in accordance with LAZ5 expression being epigenetically controlled by the LAZ2 histone H3 methyltransferase (Palma et al., 2010). The mechanism by which the loss of ACD11 triggers LAZ5 is not known, however cellular perturbations caused by the loss of ACD11 may resemble those caused by a pathogen attack monitored by LAZ5 (Palma et al., 2010).

**Paranoid plants – MPK4, PAT1 and SUMM2**

*Arabidopsis* MEKK1, MKK1/MKK2, and MPK4 form a signaling cascade that plays critical roles in plant immunity (Asai et al., 2002). This MPK pathway further negatively regulates SUMM1 (suppressor of *mkk1 mkk2*), also known as MEKK2, which in turn can activate the NLR SUMM2 (Zhang et al., 2012). Loss-of-function of MEKK1, MKK1/MKK2, or MPK4 leads to activation of defense responses (Suarez-Rodriguez et al., 2007). The autoimmune phenotypes of *mekkl, mkkl mkk2*, and *mpk4* mutants are dependent on the SUMM2, suggesting that the MEKK1-MKK1/MKK2-MPK4 cascade is monitored by SUMM2 (Zhang et al., 2012). It is thus likely that SUMM2 can sense disruption of the cascade by pathogen effectors (Zhang et al., 2012) (Figure 26).
In line with this, several studies report bacterial effectors targeting the MPK cascade (Zhang et al., 2007; Zhang et al., 2012). Interestingly, loss-of-function of \textit{summ2} completely suppresses the \textit{mkk1 mkk2} phenotype as well as the upstream \textit{mekk1}. However, the \textit{mpk4} autoimmune phenotype is only partially suppressed by \textit{summ2} (Zhang et al., 2012). This suggests that MPK4 is involved in other pathways independent of SUMM2 and that MPK4 may be guarded by additional NLRs. Furthermore, SUMM2 apparently does not interact directly with the kinase components of the MEKK1/MKK1- MKK2/MPK4 signaling cascade. It is thus likely that SUMM2 guards a downstream target of MPK4 activity.

As mentioned above MPK4 was recently connected to mRNA decapping and thus decay through its interactions with PAT1 (Roux et al., 2015). Interestingly \textit{pat1} mutants exhibit autoimmune phenotypes like \textit{mpk4}. Furthermore, the \textit{pat1} constitutive defense phenotypes are suppressed by loss-of-function of SUMM2, supporting the connection between MPK4 and PAT1 (Roux et al., 2015). Since SUMM2 deficiency only partially rescues \textit{mpk4} mutants, it is possible that this partial rescue represents a SUMM2 PAT1 branch in a MPK4 pathway (Roux et al., 2015; Zhang et al., 2012) (Figure 27). Thus PAT1 may be under surveillance by SUMM2 because it is targeted by effectors due to having a specific yet unclarified role in immunity. The connection between MPK4, PAT1 and SUMM2 is described in more detail in “mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2”.

**Figure 26.** SUMM2 activity is regulated by the MPK cascade. Detection of PAMPs leads to activation of the MPK cascade resulting in phosphorylation of MKS1 and activation of WRKYs. Furthermore phosphorylation of MPK4 inhibits the function of MEKK2 and the NLR SUMM2. Disruption of the MPK cascade results in derepression of MEKK2 and thus activation of SUMM2.

**Figure 27.** Under normal conditions, when the MPK cascade functions properly, PAT1 is phosphorylated by MPK4 and the NLR SUMM2 is kept in an inactive state. Disruptions of the MPK cascade or modification of PAT1, by either effectors or mutations, leads to activation of SUMM2 and immunity.
Guarded or not? – camta3 autoimmune mutants

Another example of NLR-induced autoimmunity involves the transcription factor CAMTA3. In *Arabidopsis*, six CAMTAs (CAMTA1-6) respond to a variety of external signals, such as wounding and drought, as well as hormonal signals like ethylene (Reddy et al., 2000; Yang and Poovaiah, 2002). CAMTA1, 2 and 3 are also important for plant tolerance to low temperature and freezing tolerance (Kim et al., 2013) and knockout of those genes significantly reduces cold tolerance (Doherty et al., 2009). CAMTA1, 2, and 3 further appear to act largely redundantly to regulate gene expression (Galon et al., 2008; Kim et al., 2013). CAMTAs have a unique CG-1 DNA binding domain (Yang and Poovaiah, 2002), varying numbers of calmodulin binding domains and a transcription factor immunoglobin domain (Bouche et al., 2002).

CAMTA3 has been shown to interact with calmodulin in a Ca\(^{2+}\) dependent manner and to bind DNA CGCG cis-elements (Yang and Poovaiah, 2002). Rapid calcium ion fluxes due to stress lead to CAMTA activation through calmodulin binding to CAMTA3 (Du et al., 2009). However, CAMTA activation and nuclear localization appears not to be strictly dependent on calmodulin (Yang and Poovaiah, 2002).

CAMTA3 is an established repressor of SA-mediated plant defenses, shown to bind the core vCGCGb motif of a number of genes including *NDR1* and *EDS1* (Doherty et al., 2009; Du et al., 2009; Nie et al., 2012). Since CAMTA3 can bind to the promoter of *EDS1*, and mutations in *EDS1* suppress the camta3 autoimmune phenotypes, CAMTA3 was proposed to function as a negative regulator of immunity and *EDS1* transcription (Du et al., 2009). In line with this, knockout of CAMTA3 leads to increased accumulation of SA and enhanced disease resistance to both bacterial (Du et al., 2009) and fungal pathogens (Nie et al., 2012), but reduced resistance against insect herbivores (Laluk et al., 2012). camta3 mutants also display dwarfism and spontaneous lesions when grown at low temperatures (Du et al., 2009).

Plants cope with environmental challenges by rapidly transducing extracellular signals into intracellular responses. This is mainly achieved by transcriptional reprogramming, initially comprised of triggering a core set of general stress response genes followed by stress-specific gene expression (Benn et al., 2014). Recently, CAMTA3 was reported to be an important integrator of the GSR through transcriptional modulation of early stress induced genes (Benn et al., 2016; Benn et al., 2014). More specifically, CAMTA3 was found to regulate the amplitude of RSRE activity in GSR (Bjornson et al., 2014). In addition camta3 autoimmunity can be suppressed by high
temperatures and mutations in \textit{PAD4} and \textit{EDS1} as well as expression of the bacterial salicylate hydroxylase NahG, implicating NLR involvement in the autoimmune phenotype.

\textbf{Uncovering the roots of paranoid suicidal plants – Aim of study and concluding remarks}

Several autoimmune mutants, like \textit{mpk4} and \textit{camta3}, were initially described as being caused by mutations in genes encoding negative regulators of cell death and/or immunity. However, more recently it has been found that the defense phenotypes of several autoimmune mutants can be suppressed by mutations in NLRs or central immune regulators, like \textit{EDS1}, \textit{PAD4} or NDR1. This indicates that the autoimmune phenotype is caused by inappropriate NLR activation. Autoimmune mutants may thus be due to loss-of-function mutations in genes encoding favored effector targets, which are guarded by NLRs. And a consequence of the guard model described above is thus that mutations in host guardees can result in NLR mediated autoimmunity. This also means that a mutation in a positive regulator of immunity may result in a phenotype masking it as a negative regulator.

To address this, a collection of ~100 site-directed putative \textit{NLR-DN} alleles was generated as a tool for mapping the roots of autoimmunity. This DN collection was generated by introducing the mismatch GXXXIGKTT to GXXXXAAT(T/S) mutation in the P-loop motif of 108 \textit{NLRs} from the \textit{Arabidopsis} ecotype Col-0. The rationale behind this approach comes from the dominant negative effects of P-loop mutations extending to most STAND family members, and observations that dominant-negative mutants may encode polypeptides that can therefore disrupt the function of wild-type gene products. Thus, dominant-negative screens by selectively inhibiting wild type NLR function with P-loop constructs may provide a powerful screening tool. In addition, this screening method may prove both more robust and timely than suppression screens of double loss-of-function mutants in searches for suppressors of autoimmune phenotypes. To search for guardees, these presumptive \textit{NLR-DN} alleles can be rapidly introduced by transformation into autoimmune mutants. Suppression of a mutant by an \textit{NLR-DN} allele will thus indicate that the corresponding protein is directly or indirectly guarded by that NLR.

We found by this DN screening approach that the autoimmune phenotype of the \textit{camta3} mutant is suppressed by DN forms of two NLRs, DSC1 and DSC2. Although \textit{camta3} mutants over-accumulate the central immune regulator \textit{EDS1} and over-expression of the \textit{CAMTA3} transcription factor was claimed to negatively regulate transcription of \textit{EDS1}, we find that \textit{EDS1} mRNA levels in \textit{camta3} mutants expressing \textit{DSC1-DN} or \textit{DSC2-DN} are equal to the levels in wild type. This
strongly suggests that the camta3 autoimmune phenotype is due to inappropriate activation of the NLRs and that CAMTA3 thus is guarded by both of these NLRs. We further show that our P-loop screening method is not only a more timely method to link NLRs to potential guardees, but is also a more robust method since our method can identify NLRs with redundant functions or working in pairs. If exploited, this collection of NLR-DNs could help us clarify the relationships between guardees and numerous ‘novel negative regulators’ of immunity and cell death in plants. This work is described in further detail in “Matching NLR immune receptors to autoimmunity in camta3 mutants using antimorphic NLR alleles”.

Future work will hopefully provide us evidence for the exact interplay between CAMTA3, DSC1 and DSC2 and further shed light on the true biological function of this guard-guardee relationship. Preliminary data indicates a role for these NLRs in resistance to the *H. arabidopsidis* isolate Noco2, however, additional work will have to be done before any conclusions can be made.

Taken together, the works described here indicates that, like CAMTA3 and MPK4, autoimmune mutants are not negative regulators per se. Although some of these autoimmune mutants may indeed be involved in regulating PTI and/or ETI, we suspect that many of them may represent favored effector targets guarded by NLRs. Thus, since the constitutive immune responses triggered by their guard may mask the true role of the guardee, their cognate NLRs will have to be ‘switched off’ in order to study the functions of these genes. This in particular for those autoimmune mutants, that is suppressed by mutations in central immune regulators, like EDS1, PAD4 or NDR1. We therefore encourage that autoimmune mutants are screened for NLR guarding before called negative regulators or until compiling evidence are shown.
**References**


Belkhadir, Y., Nimchuk, Z., Hubert, D.A., Mackey, D., and Dangl, J.L. (2004a). Arabidopsis RIN4 negatively regulates disease resistance mediated by RPS2 and RPM1 downstream or independent of the NDR1 signal modulator and is not required for the virulence functions of bacterial type III effectors AvrRpt2 or AvrRpm1. The Plant cell **16**, 2822-2835.


Dodds, P.N., Lawrence, G.J., and Ellis, J.G. (2001). Six amino acid changes confined to the leucine-rich repeat beta-strand/beta-turn motif determine the difference between the P and P2 rust resistance specificities in flax. The Plant cell 13, 163-178.


The mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2

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Abstract

Multi-layered defense responses are activated in plants upon recognition of invading pathogens. Transmembrane receptors recognize conserved pathogen-associated molecular patterns (PAMPs) and activate MAP kinase cascades, which regulate changes in gene expression to produce appropriate immune responses. For example, Arabidopsis MAP kinase 4 (MPK4) regulates the expression of a subset of defense genes via at least one WRKY transcription factor. We report here that MPK4 is involved in PTI downstream of FLS2 (Asai et al., 2002; Boller & Felix, 2009). MPK4 phosphorylation is exemplified by the binding of the bacterial flagellin-derived flg22 peptide to the leucine-rich repeat-receptor-like kinase flagellin sensing 2 (FLS2) (Gomez-Gomez et al., 2001; Chinchilla et al., 2006). PAMP recognition initiates downstream signaling, including production of reactive oxygen species, calcium influx, MAP kinase activation and global changes in gene expression that induce PAMP-triggered immunity (PTI) (Chisholm et al., 2006; Zipfel, 2009). Adapted pathogens have evolved effector proteins that are delivered into host cells to compromise PTI by evading PAMP detection or suppressing defense responses. In the second layer of immunity, plant resistance (R) proteins have evolved to directly or indirectly recognize the activities of pathogen effectors (Jones & Dangl, 2006). In the best-studied examples, R proteins are found to guard host proteins or complexes (guardians) manipulated by specific pathogen effectors. Pathogen detection via R proteins leads to induction of strong defenses and to a form of host programmed cell death known as the hypersensitive response (HR) to sequester infections. These responses are collectively termed effector-triggered immunity (ETI) (Jones & Dangl, 2006).

Changes in phosphorylation are important regulatory mechanisms in cellular signaling. Activation of mitogen-activated protein (MAP) kinase occurs within 10 min of PAMP application (Asai et al., 2002; Boller & Felix, 2009) and relies on sequential phosphorylations between MAPKK kinases (MEKK), MAPK kinases (M KK) and MAP kinases (MPK) (Pitzschke et al., 2009; Andréasson & Ellis, 2010; Rasmussen et al., 2012). In Arabidopsis, several MPKs are activated by PAMPs. A cascade comprising MEKK1-MPK4/5-MPK3/6 was initially found to be involved in ETI downstream of FLS2 (Asai et al., 2002; Droillard et al., 2004; Gao et al., 2008). Similarly, flg22 treatment activates another cascade including MEKK1, MKK1/2 and MPK4 (Petersen et al., 2000; Ichimura et al., 2006; Mészáros et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008a).

Introduction

Plant innate immunity employs multilayered defense responses comprised of two overlapping mechanisms. In the first layer, plant pattern recognition receptors detect invading microorganisms by the presence of conserved pathogen-associated molecular patterns (PAMPs) (Boller & Felix, 2009). PAMP recognition is exemplified by the binding of the bacterial flagellin-derived flg22 peptide to the leucine-rich repeat-receptor-like kinase flagellin sensing 2 (FLS2) (Gomez-Gomez et al., 2001; Chinchilla et al., 2006). PAMP recognition initiates downstream signaling, including production of reactive oxygen species, calcium influx, MAP kinase activation and global changes in gene expression that induce PAMP-triggered immunity (PTI) (Chisholm et al., 2006; Zipfel, 2009). Adapted pathogens have evolved effector proteins that are delivered into host cells to compromise PTI by evading PAMP detection or suppressing defense responses. In the second layer of immunity, plant resistance (R) proteins have evolved to directly or indirectly recognize the activities of pathogen effectors (Jones & Dangl, 2006). In the best-studied examples, R proteins are found to guard host proteins or complexes (guardians) manipulated by specific pathogen effectors. Pathogen detection via R proteins leads to induction of strong defenses and to a form of host programmed cell death known as the hypersensitive response (HR) to sequester infections. These responses are collectively termed effector-triggered immunity (ETI) (Jones & Dangl, 2006).

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Interestingly, Flg22-induced activation of MPK3, MPK4 and MPK6 is dependent on MKK1, while MPK3 and MPK6 are also activated by MKK4 (Mézéiros et al., 2006). Thus, FLS2 activates two cascades, one with an unknown MEKK and MKK4/5-MPK3/6, the other with MEKK1-MKK1/2-MPK4. More recently, the closest homologue of MPK4, MPK11, was shown to also be activated by PAMPs (Bethke et al., 2012).

*mpk4* mutants were originally found to exhibit autoimmunity, and MPK4 thus appeared to function genetically as a negative regulator of defense responses (Petersen et al., 2000; Droillard et al., 2004). However, MPK4 is activated in response to pathogens and PAMP elicitation (Droillard et al., 2004; Teige et al., 2004; Ichimura et al., 2006; Brader et al., 2007; Qiu et al., 2008a) which is counterintuitive for a negative regulator. Subsequently, it was shown that activated MPK4 interacts with and phosphorylates MAP kinase substrate 1 (MKS1), bringing about the release of the transcription factor WRKY33 and induction of the expression of the *Phytoalexin Deficient 3* (*PAD3*) gene required for biosynthesis of the antimicrobial camalexin (Andreasson et al., 2005; Qiu et al., 2008b). This illustrates how MPK4 functions as a positive regulator of PTI. Since it was recently reported that MPK4 is a target for manipulation by pathogen effectors (Zhang et al., 2007, 2012), and as *mpk4* mutant phenotypes are partially suppressed by mutations in the R protein SUMM2 (suppressor or 5-methylguanosine triphosphate cap (m7G) and the 3'-poly(A) tail. To achieve fine control of transcript abundance, mRNA is attacked by a decapping complex that breaks down RNA in a coordinated manner. mRNA decay is initiated by a decapping complex that breaks the 5'-m7G cap, and the exoribonuclease XRN that degrades the monophosphates including the 5'-m7G cap, and the exoribonuclease XRN4 (Olmedo et al., 2006; Potuschak et al., 2006; Gregory et al., 2008; Rymarquis et al., 2011; Vogel et al., 2011). It is thought that DCP1, DHH1 and DCP5 form mRNPs for translational repression of target mRNA, which are then subject to decaying by recruitment of DCP2 and VCS and digestion by XRN4 (Xu & Chua, 2009, 2011). Importantly, although PAT1 functions have not previously been studied in plants, three homologues are encoded in the *Arabidopsis* genome, each of which contains a conserved C-terminal domain. The decapping activator Sm-like (LSM) proteins, which interact with PAT1 in eukaryotes (Salgado-Garredo et al., 1999; Bonnerot et al., 2000; Bouveret et al., 2000; Tharun et al., 2000; Tharun, 2009), have recently been characterized in *Arabidopsis* (Perea-Resa et al., 2012; Golisz et al., 2013). It was found that LSM1-7 proteins form a complex and lsm1 mutants accumulate capped mRNA. Furthermore, VCS, DHH1 and PAT1 homologs were identified in LSM1 immunoprecipitates (Golisz et al., 2013).

The decapping complex plays important roles in eukaryotic development. In contrast, links between mRNA decapping and stress signaling are just being uncovered (Jiao et al., 2010; Buchan et al., 2011; Munchel et al., 2011; Park et al., 2012), and how decapping may be involved in regulating immune systems is largely unknown. Here, we characterize the *Arabidopsis* homologue of the mRNA decay regulator PAT1. We show that PAT1 functions in decapping of mRNA. Furthermore, PAT1 is phosphorylated in response to flg22 and localizes to discrete, punctate foci in the cytosol. PAT1 also interacts with MPK4 and with the R protein SUMM2 *in planta*, and the absence of PAT1 triggers SUMM2 dependent immunity. This indicates that PAT1 is regulated by MPK4 in a pathway whose disruption leads to ETI via SUMM2.

**Results**

AtPAT1 is an ScPAT1 orthologue and interacts with MPK4 *in planta*

We previously conducted a yeast two-hybrid screen to identify *Arabidopsis* proteins that interact with MPK4 (Andreasson et al., 2005). In addition to the MPK4 substrate MKS1, we identified two clones encoding PAT1 (At1g79900), an mRNA decapping stimulator involved in post-transcriptional gene regulation (Coller & Parker, 2005). Two PAT1 homologs are encoded in the *Arabidopsis* genome (*ATPAT1H1 At3g22270; ATPAT1H2 At4g14990*, Supplemental Fig S1A). The steady-state expression level of PAT1 and the homologs compared to the housekeeping gene ACTIN8 (At1g49240) was analyzed by RT-PCR (Supplemental Fig S1B). We focused on PAT1 as the other two homologs were not identified by yeast two-hybrids. To confirm the PAT1-MPK4 interaction *in planta*, doubly transgenic *Arabidopsis* lines were generated in the Ler *mpk4-1* background that expressed PAT1 with a C-terminal Myc tag and HA-tagged MPK4 under the control of their own promoters. Anti-Myc immunoprecipitation from either MPK4-HA or double transgenic MPK4-HA/Pat1-Myc tissue detected a 50 kDa band corresponding to MPK4-HA only in double transgenic lines (Fig 1A). Thus, MPK4 and PAT1 can be found in complex in *Arabidopsis*. 

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594

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mRNA decapping component PAT1 in immunity

Milena Edna Roux et al
Yeast PAT1 engages with translating mRNPs and is involved in translational repression and decapping activation (Marnef & Stan-dart, 2010). Since the function of PAT1 in Arabidopsis was unknown, we examined whether it functions similarly to yeast PAT1. To this end, a full-length Arabidopsis PAT1 cDNA was cloned from Col-0 (Supplementary Fig S1C and D) and transformed into wild-type yeast (B4742) and a yeast mutant (Y15797) in which yeast PAT1 was replaced with a G418 resistance cassette (BY4742 (YCR077c) pat1D::KanMX). In contrast to the wild-type, yeast lacking PAT1 (pat1D) display a temperature-sensitive phenotype and are impaired at 37°C but grow normally at 30°C (Tharun et al, 2005). This phenotype is reverted to wild-type in yeast containing Arabidopsis PAT1, as growth at 37°C was restored (Fig 1B). As an additional control, we transformed Arabidopsis PAT1 into wild-type
yeast (B4742/AtPAT1), and this grew similarly to wild-type at 30°C and almost as well at the wild-type at 37°C (Fig 1B). The expression of Arabidopsis PAT1 in yeast was confirmed by anti-PAT1 immunoblotting of yeast protein extracts (Supplementary Fig S1E). This provides compelling evidence for the orthologous functions of these yeast and Arabidopsis PAT1 proteins. As PAT1 is found in complex with MPK4, these results provide a link between MPK4 and post-transcriptional regulation of mRNA stability.

We next analyzed the interaction between PAT1 and conserved components of mRNA decapping. PAT1-LSM1-7 complexes function in mRNA decapping and deadenylylation (Bouveret et al, 2000; Tharun, 2009; Haas et al, 2010; Ozgur et al, 2010; Totaro et al, 2011). We therefore transiently expressed in Nicotiana benthamiana LSM1-GFP and PAT1-HA and then immunoprecipitated LSM1 with GFP Trap beads. PAT1-HA could be detected in LSM1 immunoprecipitates but did not adhere to GFP Trap beads in the absence of LSM1-GFP (Fig 1C). This is consistent with the detection of peptides corresponding to PAT1 and its homologues in LSM1 immunoprecipitates (Golisz et al, 2013) and supports a role of PAT1 in mRNA decapping. In other organisms, interactions between PAT1 and LSM1 are robust, while those between PAT1 and other mRNA decapping proteins, including the DCP1-DCP2 complex and XRNI, are more transient (Bouveret et al, 2000; Nissan et al, 2010; Ozgur et al, 2010). This is consistent with our difficulty in detecting DCP1 in complex with PAT1 in Arabidopsis (Supplementary Fig S1F).

**PAT1 is required for decapping of selected mRNAs**

In order to determine whether PAT1 behaves as an activator of mRNA decapping, we used 5' RACE to compare the levels of capped mRNAs in Col-0 and pat1 mutants. To this end, we identified an allele, pat1-1 (Salk_040660), with a T-DNA insertion in the last exon of PAT1 (Supplementary Fig S1C). We also generated an anti-PAT1 antibody against a C-terminal peptide (Supplementary Fig S1D). Immunoblotting of Col-0 protein extracts with this antibody detected a clear band around 90 kDa. In contrast, no protein could be detected in pat1-1 mutant extracts (Supplementary Fig S1G). This indicates that pat1-1 harbors either a truncated version of PAT1, no PAT1 protein, or levels of the protein that are below detection.

5' RACE was performed on transcripts known to be degraded by the decapping complex (EXPL1; UGT87A2) (Perea-Resa et al, 2012), as well as a housekeeping transcript EIF4A1. We found that capped EXPL1 and UGT87A2 accumulated in pat1-1 mutants, while capped EIF4A1 mRNA was present in equal amounts in Col-0 and pat1-1 (Fig 1D). This indicates that PAT1 plays a role in mRNA decay via decapping.

**Table 1. Phosphopeptides identified in PAT1-GFP IPs by mass spectrometry analysis.**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Phospho-sites*</th>
<th>m/z</th>
<th>Mascot score</th>
<th>PAT1GFP water</th>
<th>PAT1GFP fig22</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSFSYVPDLLPQISDQF</td>
<td>1 (S208)</td>
<td>950,93005</td>
<td>71</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>SSFSYVPDLLPQISDQF</td>
<td>2 (S208, S200)</td>
<td>990,91333</td>
<td>46</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>SSFLNYDGMLGFGDGLR</td>
<td>1 (S343)</td>
<td>929,97323</td>
<td>70</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>SSFLNYDGMLGFGDGLR</td>
<td>2 (S343, S342)</td>
<td>969,85663</td>
<td>39</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

*Phospho-sites refers to the number and in brackets the location of the phosphorylation detected by MS analysis. Potential phosphorylation sites are indicated in bold letters. m/z refers to the mass-to-charge ratio of the tabulated peptides.

**PAT1 is an MPK4 substrate**

Since MPK4 and PAT1 are found in complexes in planta, we asked whether PAT1 is an MPK substrate. PAT1 contains 5 Ser-Pro (SP) motifs which are commonly phosphorylated by MPKs (Pearson et al, 2001; Ubersax & Ferrell, 2007) (Supplementary Fig S1D). To characterize PAT1 phosphorylation in vivo, we identified PAT1 phosphopeptides by mass spectrometry. Since MPK3/4/6 are activated by flagellin and by virulent strains of Pseudomonas syringae pv. tomato (Pto) DC3000 (Asai et al, 2002; Brader et al, 2007; Suarez-Rodriguez et al, 2007; Bethke et al, 2009, 2012; Rasmussen et al, 2012), PAT1 was immunoprecipitated from extracts of untreated control and flg22-treated wild-type Col-0 or PAT1-GFP transgenic lines. Bands corresponding to PAT1-GFP (130 kDa) were excised from the gel (Supplementary Fig S2), subjected to in-gel tryptic digestion, and peptides were extracted. Phosphopeptides were enriched by TiO2 chromatography and analyzed by liquid chromatography (RP-HPLC) coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). This identified several phosphopeptides from PAT1-GFP IPs that were not detectable in the negative control (Col-0) (Table 1; Supplementary Fig S2). The most abundant phosphopeptide, based on the extracted ion chromatogram from the LC-MS/MS analysis, revealed phosphorylation of Ser208 in an SP motif (Fig 2A). Another peptide was identified with phosphorylation of Ser343. However, this site is not within an SP motif, making it a less likely site for phosphorylation by MPKs. Importantly, both these peptides have previously been detected in Arabidopsis by mass spectrometry (Phosphat Database, http://phosphat.mpimp-golm.mpg.de/). It should be noted that the PAT1 phosphopeptides were detected both whether or not the sample had been treated with flg22. Thus, under the conditions used here, PAT1 was phosphorylated and remained so after exposure to flg22.

To determine whether PAT1 is a substrate of a specific MPK, we carried out in vitro kinase assays using immunoprecipitated, PAMP-activated MPKs with purified His-PAT1 protein. Phosphorylated Hise-PAT1 was detectable as a radioactive band around 95 kDa after incubation with flg22-activated MPK4 (Fig 2B), while MPK6 caused only low levels of PAT1 phosphorylation and MPK3 did not significantly alter PAT1 phosphorylation (Supplementary Fig S3). Each MPK was also incubated with MPK4 and MPK6 IPs, and this mutant version of His-PAT1 was detectable as a radioactive band around 95 kDa after incubation with flg22-activated MPK4 (Fig 2B), while MPK6 caused only low levels of PAT1 phosphorylation and MPK3 did not significantly alter PAT1 phosphorylation (Supplementary Fig S3).
had significantly lower levels of phosphorylation (Fig 2B). This supports the identification of S208 as a key phosphorylation site in PAT1.

**pat1 mutants exhibit autoimmunity similar to mpk4**

Given that MPK4 is an important immune regulator in *Arabidopsis* and that *mpk4* mutants have de-repressed defense responses, we examined whether PAT1 may also be involved in immunity. The *pat1-1* mutant has a distinct leaf serration phenotype and a slightly smaller rosette than Col-0 (Fig 3). A similar rosette phenotype was seen in another T-DNA insertion line (*pat1-2*, WiscDSLox_734_D04 in Supplementary Figs S1C and S4), indicating that this phenotype is due to loss-of-function of PAT1. Importantly, however, the phenotype of *pat1-1* is not as extreme as *mpk4-2*, which is much smaller and has more pronounced leaf curling and reduced fertility as well as constitutive defense gene expression (Petersen et al., 2000; Fig 3).

In order to determine whether *pat1-1* is a constitutive defense mutant similar to *mpk4*, quantitative reverse-transcription PCR (qRT-PCR) was used to measure the steady-state level of the pathogenesis-related *PR1* and *PR2* genes in adult plants (Fig 4A). Previous work showed that the enhanced defense response of *mpk4* mutants is suppressed by mutations in EDS1 (Brodersen et al., 2006). Thus, *eds1-2* was crossed with *pat1-1* to explore the *pat1* phenotype in the absence of this regulator (Fig 3). Compared to Col-0, *pat1-1* mutants accumulated 1,000-fold more *PR1* and 150-fold more *PR2* transcripts (Fig 4A). In contrast, the levels of *PR* mRNAs in *pat1-1 eds1-2* double mutants were similar to those in wild-type (Fig 4A). Under the same conditions, *mpk4-2* mutants accumulated 4,000-fold more *PR1* and 800-fold more *PR2* transcripts (Fig 4A).

We found that the elevated *PR* gene expression in *pat1-1* correlated to an enhanced resistance to infection by syringe-infiltrated *Pto DC3000* when compared to Col-0 (Fig 4B). While *Pto DC3000* growth reached 5.5 cfu/cm² in Col-0, *pat1-1* mutants supported tenfold lower accumulation of *Pto DC3000*. In this experiment, *eds1-2* mutants showed enhanced bacterial growth as expected for this mutant with compromised defense responses (Fig 4B). *pat1* disease resistance is EDS1 dependent, as bacterial growth *pat1-1 eds1-2* double mutants was similar to that in *eds1-2* (Fig 4B). These findings indicate that, similar to *mpk4*, *pat1* mutants express EDS1-dependent autoimmunity in the absence of microbes.

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**Figure 2.** PAT1 is phosphorylated *in planta* and *in vitro*.

A Tandem MS spectrum of the phosphopeptide SSFSYPGSGISPDPQR in which the underlined serine is phosphorylated. Y- and b-ions are indicated that localize phosphorylation to the SP site.

B MPK4 and MPK6 were immunoprecipitated from extracts of Col-0 seedlings treated with 200 nM fig22 for 10 min. For the negative control (−), extracts were incubated with agarose beads without antibodies. IPs were incubated with His–PAT1, His–PAT1 S208A or MBP for 60 min at 37°C before boiling and SDS-PAGE. Autoradiogram (top panel), Coomassie-stained gel for loading control (bottom).

Source data are available online for this figure.
PAT1 detection in P-bodies is induced by PAMPs

Processing bodies (PBs) are cytoplasmic granules involved in both mRNA decay and translational repression pathways (Kulkarni et al., 2010). PAT1 is a conserved PB component in yeast (Rodriguez-Cousino et al., 1995), C. elegans (Boag et al., 2008), Drosophila (Mamei et al., 2010) and mammals (Scheller et al., 2007) and PBs can be significantly induced in Arabidopsis by hypoxia and heat stress (Weber et al., 2008). To investigate whether PAT1 is found in PBs, we produced transgenic Arabidopsis lines that express PAT1-GFP from its native promoter complementing the pat1 phenotype (Supplementary Fig S5A and B). We detected GFP signal in small numbers of distinct foci by confocal microscopy in roots of young seedlings (Fig 5A). Within 20 min of flg22 treatment, a significant increase in the number of GFP-positive foci could be seen in the root tips (Fig 5A). To test whether these foci correspond to PBs, we treated the roots with cycloheximide in DMSO which is known to abrogate PB formation in plants (Goeres et al., 2007). This revealed that cycloheximide inhibited flg22-induced foci (Fig 5A). Importantly, the control DMSO treatment did not reduce the number of foci (Supplementary Fig S6A and B). As a control, we also tracked the localization of the known decapping component VCS (Xu et al., 2006). Similar to PAT1-GFP, VCS-GFP was seen in flg22-induced PBs and absent when treated with cycloheximide (Fig 5B). Interestingly, PAT1 protein, which is hardly detectable under steady-state conditions, also accumulated in response to flg22 treatment, with a peak by 60 min, and a return to normal levels after 2 h (Fig 5C). PAT1-GFP mirrors this effect and was similarly up-regulated in response to flg22 treatment, as detected by anti-GFP Western blotting of seedling protein (Fig 5D). Importantly, PAT1 transcript levels were not highly induced by flg22 treatment in Col-0 seedlings (Supplementary Fig S6C), suggesting that PAT1 induction occurs post-transcriptionally. These data indicate that activation of PTI leads to up-regulation of PAT1 protein levels by an unknown post-transcriptional mechanism, and this facilitates the detection of PAT1 in PBs. In addition, PTI could induce PB formation as part of cellular reprogramming, and PAT1 may localize to PBs to engage in this process.

The MPK4 suppressor summ2 also suppresses the pat1 resistance phenotype

Autoimmunity caused by loss of different components of the MPK4 kinase cascade can be suppressed by mutations in the resistance protein SUMM2 (Zhang et al., 2012). An explanation for this is that SUMM2 keeps this PAMP responsive pathway under surveillance and that mutations in its components mimic the effects of microbial effectors that prevent phosphorylation within or below the cascade (Zhang et al., 2007). To further probe the connection between MPK4 and PAT1, we generated pat1-1 summ2-8 double mutants. These mutants retained the leaf serration phenotype of pat1-1 single mutants (Supplementary Fig S7A). However, double mutants no longer accumulated excessive PR1 and PR2 transcripts (Fig 6A) and displayed summ2 levels of susceptibility to syringe-infiltrated Pto DC3000 (Fig 6B). Importantly, the pat1 growth phenotype was not caused by overexpression of SUMM2 transcripts, as the level of SUMM2 in pat1-1 was similar to wild-type (Supplementary Fig S7B). To test whether the accumulation of 5' capped transcripts in pat1-1 (Fig 1D) was merely an effect of inappropriate activation of SUMM2, we tested the accumulation of 5' capped UGT87A2 in an XRN1 sensitivity assay. XRN1 degrades all uncapped RNA leaving 5' capped RNA intact (Blewett & Goldstrohm, 2012). The accumulation of 5' capped versus uncapped UGT87A2 transcripts was at similar levels in pat1-1 and pat1-1 summ2-8 (Fig 6C). Col-0 and summ2-8 plants had similarly reduced 5' capped versus uncapped ratios but, importantly, these were much lower than the levels of pat1-1 and pat1-1 summ2-8 (Fig 6C). Therefore, the accumulation of capped transcripts in pat1-1 mutants is not an artifact of defense activation but reflects a role for PAT1 in mRNA decay. In order to determine whether the SUMM2-mediated resistance in pat1 mutants is specific to pathogens of a specific class, we compared susceptibility to pathogens with different lifestyles. Pto DC300 is a hemi-biotrophic
mRNA decapping component PAT1 in immunity

Discussion

In this study, we identify the Arabidopsis decapping component PAT1 and show it to be an interactor and substrate of MPK4 in plant innate immunity. Furthermore, we demonstrate that Arabidopsis PAT1 complements yeast pat1Δ mutants, indicating that the function of this conserved protein is maintained in plants. The accumulation of capped mRNAs in pat1 mutants is consistent with a role for PAT1 in the mRNA decapping pathway.

Pat1 mutants exhibit a distinct leaf serration phenotype (Supplementary Fig S4) resembling those of miRNA-loss-of-function mutants (Nikovics et al., 2006) such as abb1-8 (Gregory et al., 2008), serrate (Grigg et al., 2005) and hypomorphic alleles of ago1 (Morel, 2002). This suggests that PAT1 may have a role connected to microRNA activity. Arabidopsis decapping mutants such as dep1, dep2 and res accumulate lower levels of certain miRNAs (Motomura et al., 2012). Mutants with a pat1-like phenotype, such as res, siso6 and ampi, have revealed new components in miRNA-mediated translational repression (Brodersen et al., 2008; Yang et al., 2012; Li et al., 2013). In Drosophila, HPat interacts with components of the miRNA machinery including AGO1 and GW182 (Baric et al., 2013). The connection between the mRNA decay activator HPat and the miRNA effector complex may provide a link to promote the transition of mRNA from translation to degradation. Although PAT1 may regulate targets of miRNA-mediated translational repression, the mechanism by which translational repression occurs in Arabidopsis is still under investigation.
We also find here that PAT1 is an MPK4 substrate and that *pat1* mutants exhibit autoimmunity as does *mpk4*. The connection between MPK4 and PAT1 is further supported by suppression of the *pat1* constitutive defense phenotype by loss-of-function of the SUMM2 R protein (Fig 6). However, SUMM2 deficiency only partially rescues *mpk4* mutants (Zhang *et al*, 2012), thus it...
possible that this partial rescue represents a SUMM2 PAT1 branch in the MPK4 pathway. Nevertheless, the pat1 constitutive defense phenotype is suppressed by summ2 such that pat1 summ2 mutants display a wild-type phenotype in response to biotrophic and necrotrophic pathogens (Fig 6; Supplementary Fig S8). Since PAT1 and SUMM2 also interact in planta (Fig 7), PAT1, or a PAT1-containing complex, is part of a pathway that includes SUMM2 as well as MPK4. It is therefore possible that PAT1 is under SUMM2 surveillance because it is an effector target with specific functions in immunity. Since pat1 summ2 mutants are not immunosuppressed, PAT1 and its homologues in Arabidopsis may function redundantly during PTI. An alternative explanation is that we simply have not yet tested a pathogen whose infection strategy could reveal a role of PAT1 in immunity.

mRNA decapping is not the only mRNA regulatory pathway characterized by constitutive defense responses. Indeed, mutants of nonsense-mediated decay including upf3-1, upf1-5 and smg7 mutants display autoimmune phenotypes (Jeong et al, 2011; Rayson et al, 2012a; Riehs-Kearnan et al, 2012; Shi et al, 2012). Their phenotypes are also suppressed by mutations in immune regulators such as eds1 and pad4 (Rayson et al, 2012b; Riehs-Kearnan et al, 2012), similar to what we find for pat1. This suggests that these components could also be under surveillance and may be suppressed by mutations in specific R genes. Recently, it was suggested that nonsense-mediated decay controls turnover of R gene mRNAs and cause autoimmunity in smg7 (Gloggnitzer et al, 2014). However, since autoimmunity in smg7 depends on a specific allele of the R protein RPS6, it is also possible that SMG7 is under surveillance. Most significantly, this suggests that plants have developed complex sensors to monitor the integrity of these pathways, which is consistent with the importance of differential gene expression in response to pathogen perception.

We detected PAT1 phosphorylation by PAMP-activated MPK4, and weakly also by MPK6 in vitro. We also identified PAT1 Ser208 and Ser343 as in planta phosphorylation sites by mass spectrometry. Since PAT1 phosphorylation was reduced in vitro when Ser208 was mutated to Ala, this site may be a key target of MPK4 (Fig 2B). Interestingly, Ser208 is conserved in Physcomitrella patens (moss), rice, the Arabidopsis PAT1 homologues, and Xenopus PATL2, but not in human or yeast PAT1 (Supplementary Fig S10). Although Ser208 corresponds to an SP site in Xenopus PATL1/PATL1b, PAT1

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Figure 6. SUMM2 is required for the constitutive defense phenotype of pat1 mutants.
A Elevated PR1 gene expression in pat1 mutants is suppressed by summ2-8. Four-week-old plants were used for RNA extraction, followed by qRT-PCR. Fold-change in PR1 (gray bars) and PR2 (black bars) expression is relative to Col-0, normalized to UBQ10. Standard error of the mean is indicated by errors bars (n = 3).
B pat1 resistance to P. syringae pv. tomato DC3000 is suppressed by summ2-8. Bacteria were syringe-infiltrated and samples taken 3 days post-infiltration. Data are log-transformed colony-forming units/cm²/leaf tissue (cfu/cm²). Standard error of the mean is indicated by errors bars (n = 3).
C 14-day-old seedlings grown on ms plates were used for RNA extraction. Next, 1 µg of RNA from each sample was treated with XRN1 (NEB) or mock treated before RT-qPCR. Data were normalized to ACT2, and transcript levels were compared between XRN1 and mock-treated RNA for each genotype. Standard error of the mean is indicated by error bars (n = 4). Statistical significance between the mean values was determined by ANOVA followed by Fisher’s LSD test.
D Phosphorylation of the PAT1 peptide SSFVSYPPPGISPDQR, which include Ser208, in Col-0 and mkk1/2/summ2 before and after fig22 treatment. The phosphorylation stoichiometry is illustrated relative to Col/PAT1 with OUt fig22 treatment. The ratios were obtained using quantitative iTRAQ mass spectrometry.
is known to be phosphorylated on Ser62 by an unknown kinase (Marnef et al., 2010). Thus, Ser208 may represent a plant-specific site or mechanism. While Ser208 is conserved in plant PAT1 orthologs, Ser342/3 is not. As Ser342/3 does not correspond to SP sites, they may be phosphorylated by kinases other than MPKs. Furthermore, we detected PAT1 phosphorylation in planta irrespective of PAMP treatment.

PAT1 phosphorylation by MPKs has not been shown in any system, although several phosphosites have been identified in human and yeast PAT1 proteins (PhosphoElm, http://phospho.elm.eu.org/ and Phosida http://www.phosida.com/databases). However, other decapping complex members are subject to stress-induced MPK-mediated phosphorylation. For example, human DCP1a is phosphorylated by c-Jun N-terminal kinase in response to stress (Rzeczkowski et al., 2011). Similarly, Ste20 phosphorylates yeast DCP2 upon glucose deprivation (Yoon et al., 2010). In both cases, phosphorylation seems only to be required for P-body formation and not for general decapping (Yoon et al., 2010; Rzeczkowski et al., 2011). Arabidopsis MPK6 was shown to specifically phosphorylate DCP1 in plants during dehydration stress (Xu & Chua, 2012). Thus, the regulation of mRNA decay machinery by MPKs during stress responses seems to be a key mechanism in plants and other organisms, although exactly how this affects mRNA turnover remains elusive.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as a control. Seeds for T-DNA insertion lines were from NASC (Nottingham, UK).
The T-DNA lines for At1g79090 (PAT1), both of which have insertions in the last exon, were Salk_040660 (here named pat1-1) and WiscDsLox347D04 (pat1-2). The T-DNA insertion in At1g12280 (SUMM2) summw2-8 (SAIL_1152A06), mkk1/2 (Zhang et al., 2012) and eds1-2 (Parker et al., 1996) have been described. Genotyping primers for newly described T-DNA lines are provided in Supplementary Table S1. Arabidopsis plants were grown in 9 × 9 cm pots at 22°C with a 16-h photoperiod, or on plates containing Murashige–Skoog (MS) salts medium (Duchefa), 1% sucrose and 1% agar with a 8-h photoperiod.

Cloning and transgenic lines

The genomic PAT1 (At1g79090) DNA sequence (without stop codon), plus 2 kb upstream from the start codon, was amplified from Col-0 genomic DNA and cloned into pENTR-D-TOPO (Invitrogen). The entry clone was recombined into pGWB513, pGWB517 and pGWB504 (Nakagawa et al., 2007) to obtain C-terminal HA-tagged constructs under the control of the 35S promoter. The expression clones were transformed into Agrobacterium tumefaciens strain GV3101 and transformed into Col-0 plants by floral dipping. Transformants were selected on hygromycin (30 μg/ml) MS agar and survivors tested for protein expression by Western blotting.

PAT1 protein purification and in vitro kinase assays

For *in vitro* experiments, PAT1 protein was purified from *E. coli*. The PAT1 cDNA was cloned into pET15b (for an N-terminal His fusion) and transformed into *E. coli* BL21 (pLysS). Protein expression was induced by overnight treatment with 0.5 mM IPTG at 18°C, added to cells at OD600 = 0.6. PAT1 protein was insoluble and thus purified from inclusion bodies using Bugbuster (Novagen). Proteins were solubilized in 6 M urea, 0.7% N-lauroylsarcosine, 100 mM Tris-HCl pH 8 and refolded overnight at 4°C in 0.88 M L-arginine, 55 mM Tris-HCl, 2 mM NaCl, 0.88 mM KCl, protease inhibitors. Protein was then dialyzed against 20 mM Tris-HCl pH 8, 100 mM NaCl using 3,500 MWCO dialysis tubing. Purified protein was concentrated using Centriprep 30K spin columns (Millipore) and then diluted with glycerol to a final concentration of 0.1 mg/ml.

For kinase assays, Col-0 plants were immersed in 200 nM flg22 for 10 min of to activate MAP kinases. MPK3, 4 and 6 were immunoprecipitated from 3 mg total protein extracted from flg22-treated tissue using 2 μg of each of their specific antibodies (Sigma) and 30 μl EZview protein A agarose beads (Sigma). Each microgram of purified myelin basic protein (MBP, Sigma) or 20 μg His6-PAT1 protein was incubated with washed MPK immunoprecipitates for 60 min at 37°C with 3 μCi c-ATP in kinase buffer (62.5 μM ATP, 100 mM Tris pH 7.5, 150 mM NaCl, 150 mM MgCl2, 10 mM EGTA, 5 mM DTT, Phosphotthosip inhibitor (Roche)). Kinase reactions were diluted with 4× SDS buffer and boiled for 5 min before loading on 12% SDS-PAGE gels. Following electrophoresis, gels were stained with Coomassie Brilliant Blue and incubated with gel drying buffer, followed by drying on a Bio-Rad gel dryer. Dried gels were exposed to a phosphor screen overnight.

Yeast transformation

Yeast strains pat1Δ (BY4742 (YCR077c) pat1Δ::KanMX) and B4742 were obtained from Euroscarf. PAT1 was cloned from *A. thaliana* Col-0 cDNA into pENTR-D-TOPO and recombined into yeast expression vector pVV215 (C-terminal HA tag, -URA selection; (Van Mullem et al., 2003)) by Gateway recombination. Yeast pat1Δ and B4742 cells were transformed using lithium acetate/polyethylene glycol according to the Clontech Yeast Protocols handbook. Transformed yeast was selected on SD-URA agar plates and streaked after 3–4 days onto fresh selection plates. Pat1Δ and wild-type-transformed yeast was grown in liquid culture overnight, and protein was extracted from pellet cells by vortexing with glass beads in 1× SDS extraction buffer. Boiled proteins were subjected to SDS-PAGE and anti-PAT1 immunoblotting. For temperature sensitivity assays, overnight cultures of wild-type, pat1Δ and PAT1-expressing transformants were plated on YPAD and grown at 30 and 37°C for 3 days.

Flg22 kinetics

Seedlings were grown on MS agar (Col-0) or MS agar containing 30 lg/ml hygromycin (Col-0/PAT1-GFP) for 5 days before being transferred to MS liquid medium in 24-well plates. After 10 days, seedlings were treated with the addition of flg22 to a final concentration of 100 nM (2 seedlings per well × 3 wells per treatment) time...
point). Seedlings were harvested at the indicated times and immediately frozen in liquid nitrogen for later RNA or protein extraction.

Semi-quantitative and qRT-PCR

Total RNA was extracted from seedlings with TRI Reagent (Sigma). RNA samples were treated with DNase Turbo DNA-free (Ambion), quantified with a NanoDrop spectrophotometer (Thermo Scientific) and reverse-transcribed into cDNA with SuperScript III reverse transcriptase (Invitrogen). For semi-quantitative reverse-transcription PCR (RT-PCR), Col-0 seedling cDNA was used as a template for PCR with primers specific for ACTIN8, PAT1, PATHI or PAT1H2 using Sigma Jumpstart REDTaq ReadyMix. PCR products (20 μl) were separated on 2% (w/v) agarose gels and visualized with ethidium bromide. Brilliant II SybrGreen master mix (Agilent) was used for qPCRs. The UBQ10 (At4g0320) gene was used for normalization. Gene expression of PR1, PR2, PAT1 and SUMM2 was measured by qPCR analysis, normalized to UBQ10 expression and plotted relative to Col-0 expression level. These experiments were repeated in three independent biological replicates, each with three technical replicates, and representative data are shown. Standard error is represented by error bars on figures, and statistical significance is indicated by letters above error bars. These are derived from one-way ANOVA with Tukey’s multiple comparison test (GraphPad Prism).

RNA extraction and RACE PCR

RNA extraction used TRI reagent and 10 μg RNA was used for 5 RACE according to instructions (First Choice RACE, Ambion). PCR was carried out on 1 μl of products from reverse transcription of capped RNA using DreamTaq polymerase (Fermentas) with 25 cycles. RACE PCR products (10 μl) were separated on 2% (w/v) agarose gels and visualized with ethidium bromide.

Quantification of capped versus uncapped transcripts

Total RNA was extracted from seedlings with NucleoSpin RNA columns (Machery-Nagel). To remove RNA with no 5' cap structure, 1 μg of total RNA was incubated with 1 unit XRN1 (New England Biolabs) or no enzyme at 37°C for 1 h. Next RNA was reverse-transcribed into cDNA with SuperScript III reverse transcriptase (Invitrogen). UGT87A2 transcript accumulation was measured by qPCR using SybrGreen master mix (Agilent) and normalized to ACT2. Calculating 5' capped versus uncapped transcripts was done by comparing transcript levels from XRN1 and mock-treated samples for the individual genotypes.

Confocal microscopy

Col-0/PAT1-GFP plants were grown on MS agar containing 30 μg/ml kanamycin and 2 mg/ml hygromycin for 5 days. Seedlings were placed on glass microscope slides with water, 1% DMSO or 100 nM flg22 for 20 min. For following cycloheximide and DMSO treatment, seedlings were removed from flg22 and placed on new glass microscope slides. Cycloheximide was used at 100 μg/ml in a 1% DMSO dilution, and the control was done with 1% DMSO. Imaging was done using a Leica SP5 inverted microscope.

Infection assays

Pseudomonas syringae pv. tomato DC3000 (Pto DC3000) strains were grown in overnight culture in Kings B medium supplemented with appropriate antibiotics. Cells were harvested by centrifugation and pellets resuspended in sterile water to OD600 ≈ 0.002. Bacteria were infiltrated with a needleless 1-mm syringe into four leaves on four plants per genotype and maintained in growth chambers for 3 days. Samples were taken using a cork-borer (6.5 mm) to cut leaf disks from four leaves per plant and four plants per genotype. Leaf disks were ground in water, serially diluted and plated on Kings B with appropriate selection. Plates were incubated at 28°C and colonies counted 2–3 days later. These experiments were repeated in three independent biological replicates, and representative data are shown.

For drop inoculation of B. cinerea (strain B05.10), 10 μl of 2.5 × 10⁹ spores/ml in Gamborg B5/2% sucrose (pH 5.6) was placed on the adaxial surface of fully expanded leaves of 4-week-old plants and sampled by harvesting 10–15 leaf disks per genotype at each time point, namely 0, 2 and 3 days. Data are the average of 3 biological replicates. Disease severity was measured as accumulation of the B. cinerea CUTINASE A transcript by qPCR relative to A. thaliana a-SHAGGY KINASE (At15g26751) (primers are according to Gachon & Saindrenan, 2004).

Transient expression in Nicotiana benthamiana

Agrobacterium tumefaciens strains were grown in LB medium supplemented with appropriate antibiotics overnight. Cultures were spun down and resuspended in 10 mM MgCl₂ to OD₆₀₀ ≈ 0.8. A. tumefaciens strains carrying PAT1-GFP and MPK4-HA or SUMM2-HA were mixed 1:1 and syringe-infiltrated into 3-week-old N. benthamiana leaves. Samples for protein extraction were harvested 2 days post-infiltration (dpi). Agrobacterium tumefaciens strains carrying either PAT1-HA, LSM1-GFP or PAT1-HA + LSM1-GFP were mixed 1:1 and syringe-infiltrated into 3-week-old N. benthamiana leaves. Samples for protein extraction were harvested 3 dpi. For BiFC, A. tumefaciens strains carrying PAT1 fused to the N-terminal part of YFP and SUMM2 or At4g21010 fused to the C-terminal part were mixed 1:1 and syringe-infiltrated into N. benthamiana leaves at an final OD₆₀₀ ≈ 0.8. Confocal microscopy on leaf disks was conducted 2 days post-infiltration under a Leica SP5 inverted microscope.

Protein extraction and immunoprecipitation in Nicotiana benthamiana

Leaves were ground in liquid nitrogen and extraction buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 10% (v/v) glycerol; 10 mM DTT; 10 mM EDTA; 1% (w/v) PVP; protease inhibitor cocktail (Roche); 0.1% (v/v) IGE PAL CA-630 (Sigma); Phosstop (Roche) added at 10 mM EDTA; 1% (w/v) PVP; protease inhibitor cocktail (Roche); 0.1% (v/v) IGE PAL CA-630 (Sigma); Phosstop (Roche) added at 10 mM EDTA] with appropriate selection. Plates were incubated at 28°C and colonies counted 2–3 days later. These experiments were repeated in three independent biological replicates, and representative data are shown.

For drop inoculation of B. cinerea (strain B05.10), 10 μl of 2.5 × 10⁹ spores/ml in Gamborg B5/2% sucrose (pH 5.6) was placed on the adaxial surface of fully expanded leaves of 4-week-old plants and sampled by harvesting 10–15 leaf disks per genotype at each time point, namely 0, 2 and 3 days. Data are the average of 3 biological replicates. Disease severity was measured as accumulation of the B. cinerea CUTINASE A transcript by qPCR relative to A. thaliana a-SHAGGY KINASE (At15g26751) (primers are according to Gachon & Saindrenan, 2004).
Arabidopsis protein extraction and immunoprecipitation for mass spectrometry analysis

Leaves from adult plants were ground in liquid nitrogen and extraction buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 10% (v/v) glycerol; 5 mM DTT; 2 mM EDTA; protease inhibitor cocktail (Roche); 0.1% (v/v) IGEPAL CA-630 (Sigma) and Phosstop (Roche)] added at 2 ml/g tissue powder. Samples were clarified by 20 min centrifugation at 4°C 13,000 rpm. Supernatants (45 ml) were adjusted to 3 mg/ml protein and incubated 4 h at 4°C with 50 lI GFPTrap-A beads (Chromotek) or anti-HA antibodies (4 lI, Santa Cruz) and 100 lI EZview protein A agarose beads (Sigma). Following incubation, beads were washed four times with IP buffer before adding 2× SDS and heating to 95°C for 5 min.

SDS-PAGE and immunoblotting

SDS-PAGE gels were prepared with either 8, 10 or 12% cross-linking. Proteins were loaded and gels run at 100–150 V for 1.5 h before electrophoresis onto PVDF membrane (GE Healthcare). Membranes were rinsed in TBS and blocked for 1 h in 5% (w/v) non-fat milk in TBS-Tween (0.1% (v/v)). Antibodies were diluted in blocking solution to 1:1,000 for overnight. Membranes were washed 3× before 1-h incubation in secondary antibodies, anti-rabbit or anti-mouse-HRP or anti-rabbit or anti-mouse AP conjugate (Promega; 1:5,000). Chemiluminescent substrate (ECL Plus, Pierce) was applied to overnight. Membranes were washed 3× 10 min in TBS-T (0.1%) before 1-h incubation in secondary antibodies, anti-rabbit or anti-mouse-HRP or anti-rabbit or anti-mouse AP conjugate (Promega; 1:5,000). Chemiluminescent substrate (ECL Plus, Pierce) was applied before exposure to film (AGFA CP-BU). For AP-conjugated primary antibodies, membranes were incubated in secondary antibodies, anti-rabbit or anti-mouse-HRP or anti-rabbit or anti-mouse AP conjugate (Promega; 1:5,000). Chemiluminescent substrate (ECL Plus, Pierce) was applied before exposure to film (AGFA CP-BU). For AP-conjugated primary antibodies, membranes were incubated in secondary antibodies, anti-rabbit or anti-mouse-HRP or anti-rabbit or anti-mouse AP conjugate (Promega; 1:5,000). Chemiluminescent substrate (ECL Plus, Pierce) was applied before exposure to film (AGFA CP-BU).

Antibodies

Polyclonal anti-PAT1 antibodies were generated by immunizing rabbits with synthetic peptides derived from the N-terminus (EQRIPDRTKLYPEPQ) and C-terminus (KRSMLGSQKTEPVLS) of PAT1. Antibodies (final bleed) were affinity-purified against the C-terminal peptide (Eurogentec). Antibody specificity was verified by immunoblotting with plant extracts derived from Col-0 and pat1-1 tissue. Mouse anti-HA and anti-GFP antibodies were obtained from Santa Cruz. Rabbit anti-GFP antibodies were obtained from AMS Biotechnology. Anti-MPK3, anti-MPK-4 and anti-MPK-6 antibodies were obtained from Sigma. Secondary antibodies were obtained from Promega.

In-gel digestion, TiO2 chromatography and mass spectrometry

Bands excised from SDS-PAGE were chopped into small pieces and incubated for 1 h in 50 mM TEAB, acetonitrile (50:50) on a shaker at room temperature. After incubation, the sample was centrifuged shortly and the supernatant was discarded. Gel pieces were dried in a vacuum centrifuge for 15 min and subsequently 30 lI of trypsin (10 ng/lI) in 20 mM TEAB pH 7.5 was added to cover the dried gel pieces. The solution was incubated in 4°C for 30 min. After incubation, the trypsin solution was replaced with 30 lI 20 mM TEAB pH 7.5 and the tube was incubated at 37°C overnight. Peptides from the digestion solution after incubation were recovered in a low binding Eppendorf tube (Sorensen Bioscience), and the gel pieces were washed with 50% acetonitrile for 15 min to extract more peptides. The washing solution was mixed with the recovered peptides, and the peptide solution was lyophilized. Phosphopeptides were purified by titanium dioxide (TiO2) chromatography (Larsen et al., 2005). Lyophilized peptides were resuspended in loading buffer for TiO2 chromatography (80% acetonitrile, 5% TFA, 1 M glycine acid), and 0.3 mg TiO2 beads (GL Science, Japan) were added to the solution and incubated for 10 min. After incubation, the beads were pelleted by centrifugation and the supernatant was removed. The beads were washed once with 80% acetonitrile, 1% TFA and once with 10% acetonitrile, 0.1% TFA. Phosphopeptides were eluted using 1% ammonium hydroxide and desalted and concentrated prior to LC-MS/MS using a Poros Oligo R3 micro-column (Engholm-Keller et al., 2012).

For quantitative phosphopeptide analysis, the tryptic peptide mixtures after in-gel digestion were labeled with iTRAQ 4 plex (according to the manufacturer’s protocol) and the samples were mixed prior to TiO2 enrichment as described above. The non-phosphorylated peptides were concentrated and desalted using a Poros Oligo R3 micro-column (Engholm-Keller et al., 2012). The non-phosphorylated peptides were used to normalize the PAT1 protein level.

LC-MSMS analysis was performed using an EASY-LC system (Proxeon, Thermo Fisher Scientific) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) as described previously (Engholm-Keller et al., 2012). Peptides were separated using a 20 min gradient from 0% to 34% B-buffer (A-buffer: 0.1% formic acid; B-buffer: 90% acetonitrile, 0.1% formic acid). The data-dependent analysis was performed using one MS full scan in the area 300–1,800 Da performed in the Orbitrap with 60,000 in resolution, followed by the five most intense ions selected for MSMS (collision induced dissociation) performed in the linear ion trap.

Raw data from the LTQ-Orbitrap-XL were processed using the Proteome Discoverer (PD) program (Thermo Fisher Scientific, Bremen, Germany). The data were searched against the Arabidopsis database (33,596 sequences; 13,487,687 residues) using the Mascot search engine. Precursor ions were searched with 10 ppm mass tolerance and 0.8 Da per peptide. The data were validated using the ‘Fixed Value PSM Validator’ in PD. All identified peptides were normalized based on the non-phosphorylated peptides.

Supplementary information for this article is available online: http://emboj.embopress.org

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mRNA decapping component PAT1 in immunity

Milena Edna Roux et al

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Author contributions

MER, MWR, KP, JM and MP conceived and designed the experiments. MER, MWR, JM and MP analyzed the data. MER, MWR, KP, JM and MP wrote the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

References


Bonnerot C, Boeck R, Lapeyre B (2000) The two proteins Pat1p (Mrt1p) and Spb8p interact in vivo, are required for mRNA decay, and are functionally linked to Pab1p. Mol Cell Biol 20: 5939 – 5946


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Rymarquis LA, Sourel FF, Green PJ (2011) Evidence that XRN1, an Arabidopsis homolog of exoribonuclease XRN1, preferentially impacts transcripts with certain sequences or in particular functional categories. RNA 17: 501 – 511


Figure S1
Figure S1: Pat1 alleles, protein and immunoblotting
A. Phylogenetic tree of PAT1 orthologues (neighbor-joining tree generated from MAFFT multiple alignment in Geneious version 5.6.6 created by Biomatters (http://www.geneious.com/). AtPAT, Arabidopsis thaliana; CePAT, C. elegans; OsPAT1, Oryza sativa; HPat, Drosophila melanogaster; HsPAT, Homo sapiens; ScPAT, Saccharomyces cerevisiae; XlPAT, Xenopus laevis.

B. RT-PCR analysis of PAT1, ACTIN8, PAT1H1 and PAT1H2 gene expression in Arabidopsis Col-0 seedlings.

C. PAT1 gene structure. Promoter (grey box), exons (white boxes) and introns (black lines). Start codon is marked ATG. T-DNA insertion sites of pat1-1 (Salk_040660) and pat1-2 (WiscDs_437D04) are marked with black triangles in exon.

D. PAT1 contains N-terminal, Pro-rich, middle and Pat-C domains. Peptides for antibody generation are indicated by black pentagons. Potential SP phosphosites are marked with black stars. Amino acid numbers are above each domain.

E. Anti-PAT1 immunoblotting of yeast protein extracts from pat1∆ transformed with AtPAT1 and untransformed pat1∆. AtPAT1 corresponds to the 89 kDa band seen in the pat1∆ transformed with AtPAT1. A nonspecific band indicated by *.

F. Co-IP between PAT1-GFP and DCP1-HA. Transgenic lines expressing DCP1-HA (1,2) or PAT1-GFP and DCP1-HA (3,4) were treated with water (-) or 200nM flg22 (+) for 15 mins. Proteins were extracted and subjected to HA and GFP IPs. Immunoblots of inputs (20 µg each, top panel), GFP IPs (middle panel) and HA IPs (bottom panel) were probed with anti-GFP and anti-HA antibodies. GFP IPs were also probed with anti-MPK4 antibodies as a control for PAT1-GFP IPs. Molecular weights indicated on the right in kDa.

G. The anti-PAT1 antibody does not cross-react with any protein in pat1-1 mutant total protein extracts. Protein extracts (35 µg) derived from wild-type Col-0 and pat1-1 probed with 1:250 dilution of anti-PAT1 and alkaline phosphatase-conjugated anti-rabbit antibodies.
Figure S2: Mass spectrometry analysis of PAT1-GFP. Colloidal Coomassie-stained SDS-PAGE of GFP IPs from Col-0 or Col-0/PAT1-GFP treated with water or flg22. Molecular weight in kDa indicated on the left.
Figure S3: MPK3 does not phosphorylate PAT1 in vitro.
MPK3 was immunoprecipitated from Col-0 seedlings treated with water (-) or 200 nM flg22 (+) for 10 minutes. MPK3 IPs were incubated with His6-PAT1 or MBP for 30 mins at 37°C before SDS-PAGE. Autoradiogram (top panel), Coomassie-stained gel for loading control (bottom). Molecular weight in kDa indicated at right.
Figure S4: Pat1 mutants have a leaf serration phenotype. 4-week-old (left) or 8-week-old (right) plants grown under short-day conditions.
Figure S5

Figure S5: PAT1-GFP complements the pat1-1 phenotype.

A. Plants photographed at 5-weeks of growth in short-day conditions where PAT1-GFP complements the pat1-1 leaf serration phenotype.

B. The accumulation of PR1 and UGT87A2 transcripts in pat1-1 are reverted when introducing PAT1-GFP under control of the native PAT1 promoter. Transcript levels were quantified by qRT-PCR using RNA extracted from 5-week-old plants grown in soil under short-day conditions. Fold change is represented relative to Col-0 and normalized to ACT2. Standard error of the mean is indicated by error bars (n=3) and statistical significance determined by ANOVA followed by Tukey’s test is shown as letters above error bars.
Figure S6: effect of flg22 treatment on PAT1 transcript and PAT1-GFP protein.
A. Confocal microscopy with root elongation zones of five-days-old Col-0/PAT1-GFP seedlings. Roots treated in DMSO show no significant change in localization or amounts of GFP signal.
B. The number of GFP foci in PAT1-GFP seedlings is clearly induced upon treatment with 1µM flg22 on glass slides for 20 min (second panel). No reduction in GFP foci is seen after treatment with DMSO for 60 min (bottom panel). The scale bar corresponds to 10 µm.
C. qRT-PCR analysis of PAT1 transcripts in Col-0 seedlings treated with time course of 100 nM flg22 with time in minutes post-treatment as indicated. UBQ10 was used for normalization. Expression is displayed as fold change (where Col-0 at t=0 mins is 1). Standard error of the mean is indicated by error bars (n=3).
Figure S7: pat1 summ2 mutant phenotype
A. 4-week-old plants grown under short-day conditions.
B. qPCR analysis of SUMM2 transcripts in 4-week-old Arabidopsis Col-0, pat1-1, summ2-8 and pat1-1 summ2-8 plants. UBQ10 was used for normalization. Expression is displayed as fold change (where Col-0 = 1). Standard error of the mean is indicated by error bars (n=3).
Figure S8: Infection of pat1-1 summ2-8 mutants with necrotrophic fungus. B. cinerea drop inoculation infection of 4-week-old Col-0, pat1-1, summ2-8 and pat1-1 summ2-8 plants sampled at 0, 2 and 3 days. Data are the average of 3 biological replicates. Disease severity was measured as accumulation of the B. cinerea CUTINASE A transcript by qPCR relative to A. thaliana α-SHAGGY KINASE (At5g26751).
**Figure S9**

Figure S9: PAT1 is associated with LSM1, MPK4 and SUMM2 in *N. benthamiana*. Proteins were transiently co-expressed (PAT1-HA with LSM1-GFP, Myc-GFP, MPK4-GFP or SUMM2-GFP) in *N. benthamiana* and tissue and harvested 2 days post-infiltration. Anti-HA immunoblot of PAT1-HA IPs are shown in the top panel. Anti-GFP immunoblot of PAT1-HA IPs are shown in the middle panels. The bottom panel show the presence of LSM1-GFP, Myc-GFP and MPK4-GFP in the input. Although we were unable to detect SUMM2-GFP in the input SUMM2-GFP was easily detected in the enriched PAT1-HA IP. Molecular weights are shown in kDa at left.
**Figure S10**

Figure S10: Multiple alignments of key regions of PAT1 homologues from several kingdoms. Alignment used MSAFT algorithm in Geneious version 5.6.6 created by Biomatters (http://www.geneious.com/). Pink boxes mark predicted SP sites; yellow boxes mark phosphorylation sites identified by MS in this study.
### Supplementary Table I: Primers used in this study

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Matching NLR immune receptors to autoimmunity in \textit{camta3} mutants using antimorphic NLR alleles

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Summary

To establish infection, pathogens deploy effectors to modify or remove host proteins. Plant immune receptors with nucleotide-binding, leucine-rich repeat domains (NLRs) detect these modifications and trigger immunity. Plant NLRs thus ‘guard’ host ‘guardees’. A corollary is that autoimmunity may result from inappropriate NLR activation because mutations in plant guardees could trigger corresponding NLR guards. To explore these hypotheses, we expressed 108 dominant-negative (DN) \textit{Arabidopsis} NLRs in autoimmune mutants and transformed this collection into various lesion mimic mutants, including \textit{camta3}, which exhibits autoimmunity. CAMTA3 was previously described as a negative regulator of immunity, and we find that autoimmunity in \textit{camta3} is fully suppressed by expressing DNs of two NLRs, DSC1 and DSC2. Additionally, expression of either NLR triggers cell death that can be suppressed by CAMTA3 expression. These findings support a model in which DSC1 and DSC2 guard CAMTA3, and suggest that other negative regulators of immunity may similarly represent guardees.

Introduction

The innate immune system includes receptors that recognize pathogen-associated molecular patterns (PAMPs). Thus, plants and animals have bacterial flagellin receptors that trigger immunity (Gomez-Gomez and Boller, 2002). Successful pathogens deliver effectors into host cells to suppress this layer of immunity (Jones and Dangl, 2006). In a next layer, cytoplasmic nucleotide-binding leucine-rich repeat (NLR) receptors, that are similar to animal NOD-like receptors, directly or indirectly recognize the activities of pathogen effectors. NLRs activate effector triggered immunity (ETI) that is often associated with local host cell death known as the hypersensitive response (HR) (Dangl et al., 2013). Two subfamilies of plant NLRs can be defined by the presence of either an N-
terminal Toll/Interleukin-1 receptor (TIR) or a coiled-coil (CC) domain (Jones and Dangl, 2006). Signaling by TIR-NLRs generally requires ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), whereas NON-RACE SPECIFIC DISEASE RESISTANCE1 (NDR1) is important for CC-NLR triggered HR (Aarts et al., 1998). So while bacterial effectors possess diverse activities to manipulate host responses (Speth et al., 2007), NLRs with diverse recognition specificities and downstream signaling are found in single plant species (DeYoung and Innes, 2006). An example of this complexity is the bacterial pathogen Pseudomonas syringae (Pst) which injects effectors via a type III secretion system to establish infection in Arabidopsis (Buell et al., 2003). These effectors include AvrRpm1 and AvrRpt2 which target the host protein RIN4 by phosphorylation or degradation respectively. These changes in RIN4 are detected by two NLRs, RPM1 and RPS2, which both trigger ETI (Axtell and Staskawicz, 2003; Belkhadir et al., 2004). Importantly, loss of RIN4 results in RPS2-dependent autoimmunity indicating that RPS2 guards RIN4 (Axtell and Staskawicz, 2003; Spoel and Dong, 2012).

Numerous autoimmune or lesion mimic mutants are caused by gain-of-function mutations in NLRs (Shirano et al., 2002; Zhang et al., 2003), or by loss-of-function in diverse genes thought to act as negative regulators of immunity and the HR (Dietrich et al., 1994; Greenberg and Ausubel, 1993; Greenberg et al., 1994; Lu et al., 2011; Petersen et al., 2000; Shirano et al., 2002; Zhang et al., 2003). Interestingly, the autoimmune phenotypes of these mutants are largely the same as those for ETI, including EDS1/PAD4 or NDR1 dependency, stunted growth, accumulation of reactive oxygen species and elevated defense gene expression (Brodersen et al., 2006; Brodersen et al., 2002; Grant et al., 2000; Sohn et al., 2014; Zhang et al., 2003). In addition, like ETI, autoimmunity can often be suppressed by high growth temperature (Zhang et al., 2012). Importantly, some 40% of Arabidopsis autoimmune mutants are suppressed by mutations in NLRs and other immune signaling components (Bruggeman et al., 2015; Rodriguez et al., 2016).

Other evidence linking autoimmunity and NLRs may be hybrid necrosis. Some 2% of intraspecific Arabidopsis crosses yield F1 progeny with hybrid necrosis or autoimmunity which can be suppressed at higher growth temperatures (Bombles and Weigel, 2007). These incompatibility loci often map to rapidly evolving NLR genes or gene clusters. Such hybrid necrosis may be due to activation of NLR dependent defense responses due to a failure in guard–guardnee interactions (Chae et al., 2014).

In some instances, NLR dependent autoimmunity is well described. ACCELERATED CELL DEATH11 (ACD11) is a ceramide-1-phosphate transfer protein (Simanshu et al., 2014), and
*acd11* mutants exhibit autoimmunity dependent on the NLR LAZ5. Thus, *acd11* is rescued by *laz5* knockout and by a dominant negative *laz5-D2* allele, indicating that ACD11 or its complexes/pathways are likely effector target(s) guarded by LAZ5 (Palma et al., 2010). The *laz5D-2* allele has a mutation in the P-loop (Ile→Asn) of its nucleotide binding domain (Palma et al., 2010). This is similar to the *DN* mutation (Val→Asn) of the tobacco NLR *N* which also abolishes its function (Mestre and Baulcombe, 2006). Clearly, the P-loop is important for NLR function.

Knockout of *MAP kinase 4 (MPK4)*, or double knockouts of the two upstream kinases *MKK1* and 2, also lead to autoimmunity which is suppressed by mutations in the *NLR SUMM2*. Since the MPK4 pathway is a target of the HopAI1 effector and HopAI1 activity triggers SUMM2, loss of MKK1/2 mimics the presence of HopAI1 and triggers SUMM2-dependent autoimmunity (Zhang et al., 2012). It is therefore likely that NLRs cause autoimmunity in plants with mutations in genes encoding effector targets.

An example of such NLR induced autoimmunity might be the CALMODULIN BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3) with 5 homologs in *Arabidopsis* (CAMTA1-6) (Bouche et al., 2002). CAMTA1, 2 and 3 appear to coordinately regulate gene expression (Kim et al., 2013), but loss of CAMTA3 is sufficient to cause autoimmunity (Galon et al., 2008). Since CAMTA3 can bind to the promoter of *EDS1*, and mutations in *EDS1* rescue *camta3* mutants, CAMTA3 was proposed to function as a negative regulator of immunity and *EDS1* transcription (Du et al., 2009). In contrast, CAMTAs may positively regulate early stress response genes via a core CAMTA binding sequence present in their promoters (Benn et al., 2014).

We describe here a collection of 108 *Arabidopsis* NLRs mutated in their P-loops to potentially create their corresponding dominant-negative *NLR-DN* alleles. We transformed this collection into various lesion mimic mutants, including *camta3*, to screen for suppression of autoimmunity. As proof-of-principle, we first showed that transgenic lines expressing *RPM1-DN* are indistinguishable from *rpm1-3* knock-out mutants in terms of ETI and gene-for-gene resistance. Importantly, *RPM1-DN* did not interfere with common CC-NLR or TIR-NLR signaling pathways. In addition, we found that *DN* mutants of two NLRs we name *DSC1* and *DSC2* fully suppress autoimmunity in the *camta3* mutant. Since DSC1 and DSC2 triggered cell death in *N. benthamiana* is prevented specifically by *CAMTA3* expression, and as DSC1 appears to interact with both CAMTA3 and DSC2, autoimmunity in *camta3* is probably not caused by a lack of its proposed function as a negative regulator of genes required for immunity. Instead, the *camta3* phenotype is triggered by the NLRs DSC1 and DSC2.
Results

Function and specificity of P-loop mutations

Screens for suppression of one or more autoimmune mutants by NLR loss-of-function mutations are time consuming and potentially uninformative for NLRs with redundant functions. We therefore took an alternative, transgenic approach with a screen for suppression in T2 transformants. Since specific mutations in the P-loop domain of NLRs can have dominant negative effects (Dinesh-Kumar et al., 2000; Palma et al., 2010; Roberts et al., 2013), we examined the consequences of a P-loop mutation in the well-studied CC-NLR RPM1 (GK,221,222,AA; here named RPM1-DN). RPM1 triggers cell death in plants infected with Pst DC3000 (AvrRpm1) (Grant et al., 1995). We compared cell death responses in 4 week old Col-0, rpm1-3 knock-out mutant, and two independent RPM1-DN transformants (RPM1-DN1 and RPM1-DN2) using an electrolyte leakage assay (Mackey et al., 2003). As expected, cell death measured as conductance increased in Col-0 already after 3 hours (Figure 1A). However, this increase was suppressed in RPM1-DN1 and RPM1-DN2 plants to the same extent as in rpm1-3 (Figure 1A). We also tested resistance responses in 4 week old, short-day-grown rosette leaves syringe-infiltrated with Pst DC3000 (AvrRpm1) by measuring bacterial growth at 0 and 3 days. After 3 days, bacterial growth was almost 100 fold higher in RPM1-DN1, RPM1-DN2 and rpm1-3 compared to Col-0 (Figure 1B). Moreover, we did not observe any difference in growth of virulent Pst DC3000 between Col-0, rpm1-3 mutants and plants expressing RPM1-DN (Figure 1C). Thus, expression of RPM1-DN in wild type plants compromises the function of RPM1.

We then tested the specificity of this dominant suppression by examining if resistance to bacteria expressing AvrRps4 and AvrRpt2 was affected by expression of RPM1-DN. AvrRps4 is recognized by the TIR-NLR pairs RPS4/RRS1 and RPS4B/RRS1B (Narusaka et al., 2009; Saucet et al., 2015) whereas resistance against AvrRpt2 is conferred by the CC-NLR RPT2 (Tsuda et al., 2013). This revealed that two RPM1-DN lines supported similar levels of growth of Pst DC3000 (AvrRps4) as Col-0 and rpm1-3, while the susceptible control eds1-2 supported significantly higher growth (Figure 2A). Similarly, expression of RPM1-DN did not interfere with recognition of AvrRpt2, as the RPM1-DN lines did not support more bacterial growth than Col-0 (Figure 2B). As expected, the susceptible control ndr1 supported significantly higher bacterial growth than the other genotypes (Figure 2B). Thus, plants expressing dominant negative versions of RPM1 are indistinguishable from rpm1-3 mutants and the function of other NLRs is unaffected. Lastly, to confirm that mutations in specific NLRs can suppress single autoimmune mutants, we mutated the
P-loop in SUMM2. Loss-of-function mutations in the de-capping activator **PAT1 (PROTEIN ASSOCIATED WITH TOPOISOMERASE II NUMBER 1)** lead to SUMM2-dependent autoimmunity including dwarfism and elevated levels of the defense marker **PATHOGENESIS RELATED 1 (PRI)** transcripts (Roux et al., 2015). Similar to *pat1 summ2* double homozygotes, expression of **SUMM2-DN** in *pat1* suppressed dwarfism, enhanced resistance and elevated **PRI** transcript levels (Figure S1). Thus, as in *pat1 summ2* plants, autoimmunity is suppressed in *pat1* mutants expressing **SUMM2-DN**.

**Introducing a P-loop mutation in multiple Arabidopsis NLRs**

The proof-of-concept with **DN** mutant forms of RPM1 and **SUMM2** prompted us to introduce such mutations into an additional 106 Arabidopsis NLRs (Table S1). These NLRs were used because they were readily cloned/mutated and because they encode ‘typical’ TIR- (72 genes) or CC-NLRs (34 genes) without truncations or additional domains. With RPM1 and **SUMM2**, this allele collection represents 89% (73/82) of Arabidopsis TIR-NLRs and 73% (35/48) of CC-NLRs (Meyers et al., 2003). More specifically, the conserved P-loop motif **GXXXXGKT(T/S)** in these genes was mutated to **GXXXXAAAT(T/S)** using mismatch primers and a multi-fragment USER cloning approach (Geu-Flores et al., 2007). Sequenced clones were introduced into Agrobacterium GV3101 and then transformed into wild type plants and a collection of autoimmune mutants including camta3. Independent T1 plants (T2 seeds) were collected after BASTA selection and screened for suppression of stunted growth, chlorosis or early flowering phenotypes exhibited by the autoimmune mutants. In a screen of ~60 **NLR-DN** expressing lines, we identified one line exhibiting suppression of the **camta3** phenotype (Figure 3A). We named this line, which expresses the DN form of the NLR encoded by *At4g12010*, **DSC1-DN** for **DOMINANT SUPPRESSOR OF camta3 NUMBER 1-DOMINANT NEGATIVE**. **DSC1** encodes a typical TIR-NLR proposed to be part of an NLR pair together with the TIR-NLR **At4g12020** (Narusaka et al., 2009). This head to head pair may share a small promoter region of 273bp and both genes are lowly expressed (AtGenExpress). However, Meyer et al. (2007) found that DSC1 is 1 of only 38 NLRs for which mRNA expression levels are affected by SA or flg22, while *At4g12020* is not. Although *At4g12020* is the closest homolog of DSC1, it contains WRKY and MAPx kinase domains and was therefore not included in our **NLR-DN** collection.

There are six CAMTA proteins in Arabidopsis, and null alleles of **CAMTA1** or **CAMTA2** in **camta3** mutants enhance the latter’s dwarfism and chlorosis (Kim et al., 2013).
However, expression of DSC1-DN in camta1 camta3 double mutants did not suppress this growth defect. This indicates that DSC1-DN suppression is specific to CAMTA3 (Figure S2).

NLR-dependent autoimmunity in camta3

T3 lines homozygous for DSC1-DN were examined for suppression in more detail. 6 week-old camta3 mutants grown in short days exhibited stunted growth, necrotic lesions in older leaves (Figure 3A) and clusters of dead mesophyll cells (Figure 3B) (Du et al., 2009). In contrast, camta3 DSC1-DN appeared wild type (Figure 3A and 3B). Expression of DSC1-DN in Col-0 did not affect its wild type growth and did not induce cell death (Figure 3A and B).

camta3 mutants also exhibit increased resistance towards virulent Pst DC3000 (Galon et al., 2008). To evaluate if this trait was also abrogated by expression of DSC1-DN, we syringe inoculated 5 week-old plants with Pst DC3000 and measured bacterial growth after 3 days. While bacterial growth in camta3 mutants was significantly lower than in Col-0, bacterial growth was restored to wild type levels in camta3 DSC1-DN (Figure 3C). Importantly, expression of DSC1-DN in Col-0 did not affect susceptibility (Figure 3C).

camta3 mutants constitutively express defense genes including PRI1 (Du et al., 2009). However, PRI1 mRNA levels were restored to Col-0 levels in camta3 DSC1-DN lines, and the expression of DSC1-DN in Col-0 did not affect PRI1 mRNA levels (Figure 3D).

Since CAMTA3 was found to bind the EDS1 promoter and was reported to be a negative regulator of EDS1 expression, elevated levels of EDS1 and other CAMTA3 regulated transcripts were thought to cause autoimmunity in camta3 (Du et al., 2009). While autoimmunity in camta3 mutants may be suppressed by DSC1-DN, expression of EDS1 should remain elevated in camta3 DSC1-DN. In agreement with previous reports, we found that EDS1 mRNA levels are significantly higher in 5 week-old camta3 compared to Col-0 plants (Figure 3E). However, the levels of EDS1 transcripts in camta3 DSC1-DN were not significantly different from those in Col-0 or DSC1-DN single mutants (Figure 3E). These results indicate that autoimmunity in camta3 mutants is triggered by DSC1 and not by the loss of negative regulation of EDS1 and other regulatory transcripts.

CAMTA3 interacts with DSC1 and inhibits DSC1 triggered HR in N. benthamiana
To further investigate the relationship between DSC1 and CAMTA3 we expressed them in *N. benthamiana* as transient over-expression of NLRs can trigger HR cell death in this system (Cesari et al., 2014). In line with this, expression of DSC1 alone triggered strong and rapid HR-like cell death (Figure 4A). This DSC1-triggered HR was suppressed by co-expression with CAMTA3 (Figure 4A), while co-expression of CAMTA1 or CAMTA2 had no influence on DSC1 triggered HR (Figure 4A). Expression of the three CAMTAs alone did not induce a reaction. DSC1 therefore acts as an HR trigger specifically in the absence of CAMTA3.

The above results suggest that DSC1 and CAMTA3 represent a guard/guardee pair. We therefore speculated that DSC1 and CAMTA3 may be found together in subcellular complexes. To assess this, we examined the localization of CAMTA3 fused to CFP (Figure S3A) and DSC1 fused to YFP (Figure S3B). Since both showed cytoplasmic and nuclear localization when transiently expressed in *N. benthamiana*, we tested their interaction *in vivo* by Förster Resonance Energy Transfer Acceptor Bleaching (FRET-AB). Using CFP_CAMTA as donor and YFP_DSC1 as acceptor we observed a clear increase in donor fluorescence (FRET efficiency) after photo bleaching of YFP (Figure 4B). The same FRET efficiency was not seen with donor/acceptor pairs of CFP_CAMTA and YFP_SUMM2 or of CFP_MPK4 and YFP_DSC1, included as negative controls (Figure 4C). To confirm the positive FRET we transiently expressed HA_DSC1 with CAMTA3_CFP or GFP_MYC in *N. benthamiana* and immunoprecipitated DSC1 with HA-trap beads. Only CAMTA3_CFP was detected in HA_DSC1 precipitates (Figure 4D), confirming that DSC1 and CAMTA3 can exist in complexes *in planta*.

**Autoimmunity in camta3 depends on two NLRs**

To further probe connections between DSC1 and CAMTA3, we generated *camta3 dsc1* double loss-of-function mutants. Surprisingly, these double mutants appeared indistinguishable from *camta3* single mutants (Figure 5A), and upon inoculation with *Pst* DC3000 they showed *camta3*-like enhanced resistance (Figure 5B). In addition, like *camta3*, *camta3 dsc1* had elevated levels of *PR1* and *EDS1* expression (Figure 5C and D). This suggests that DSC1-DN interferes with more than just DSC1 function. As two or more NLRs may guard the same guardee (Belkhadir et al., 2004; Eitas et al., 2008), and as NLRs may dimerize (Eitas and Dangl, 2010; Narusaka et al., 2013; Narusaka et al., 2009), we speculated that DSC1 might function together with another NLR. If so, DSC1-DN might poison their cooperativity while the absence of DSC1 in the *dsc1* mutant would not. We therefore screened our remaining NLR-DN alleles for suppression of *camta3* autoimmunity.
and found an NLR-DN allele of At5g18370, here called DSC2-DN, whose expression also fully suppressed the camta3 phenotypes (Figure 6A). The closest DSC2 homolog (At5g18350), separated from it by ~10kb encoding both a TIR-NLR (At5g18360) and another gene (At5g18362), was included in the NLR-DN screen but did not exhibit suppression of camta3.

As for DSC1-DN, expression of DSC2-DN in camta3 abrogated resistance to Pst DC3000 (Figure 6B) and restored PRI expression almost to wild type levels (Figure 6C). Expression of DSC2-DN in Col-0 did not affect resistance or PRI expression levels. Importantly EDS1 mRNA levels in camta3 DSC2-DN were similar to those in Col-0 and DSC2-DN (Figure 6D). We also found that, as for DSC1, transient expression of DSC2 in N. benthamiana triggered HR-like cell death which was suppressed by co-expression with CAMTA3 (Figure 6E). This again indicates that autoimmunity in camta3 mutants is triggered by NLRs and is not due to the loss of CAMTA3 as a negative regulator of EDS1.

We generated camta3 dsc2 double mutants to further examine the connection between CAMTA3 and DSC2. Unlike camta3, camta3 dsc2 did not exhibit dwarfism, but leaf development was not restored to wild type (Figure S4A). camta3 dsc2 mutants also had only partial suppression of resistance toward Pst DC3000 (Figure S4B), and showed higher expression of PRI and EDS1 compared to wild type (Figure S4C and D).

Since camta3 dsc1 showed no suppression, and camta3 dsc2 showed only partial suppression of the camta3 autoimmune phenotypes, we generated triple camta3 dsc1 dsc2 mutants. These triple mutants appeared wild type and developed like Col-0 (Figure 7A), while camta3 growth defects were restored in the triple mutant complemented with genomic clones of either DSC1 or DSC2 (Figure S5). Although the triple mutants did not develop visible autoimmune phenotypes, it was possible that they retained increased pathogen resistance. To test this, we inoculated leaves of Col-0, camta3 and camta3 dsc1 dsc2 with Pst DC3000. This demonstrated that camta3 dsc1 dsc2 resistance was restored to wild type levels (Figure 7B). While increased resistance was seen in camta3 and in camta3 dsc1 and camta3 dsc2 3 days post inoculation (Figures 5B and S4B), bacterial growth in camta3 dsc1 dsc2 reached the same levels seen in Col-0 and in camta3 DSC1-DN and camta3 DSC2-DN (Figures 7B, 3B and 6B). In addition, PRI levels were restored to wild type levels in the camta3 dsc1 dsc2 triple mutant (Figure 7C), in contrast to the higher levels in camta3 dsc1 and camta3 dsc2 (Figures 5C and S4C). Furthermore, EDS1 mRNA levels in camta3 dsc1 dsc2 plants were similar to those in Col-0 (Figure 7D).
In summary, DSC1 and DSC2 contribute to autoimmunity in camta3. Although they seem to operate independently, the fact that the dominant negative version of either one influences the other suggests that DSC1 and DSC2 interact directly or indirectly. To test this we transiently expressed HA_DSC1 with DSC2_YFP, YFP_DSC2 or GFP-MYC in N. benthamiana and immunoprecipitated DSC2 with GFP-trap beads. As a specificity control we also expressed DSC2-YFP, YFP-DSC2 and GFP-MYC with HA-RPS4 (Zhang et al., 2004) as DCS1 and RPS4 share 61% amino acid identity. HA_DSC1 could only be detected in DSC2 precipitates and not in GFP_MYC precipitates (Figure 7E). In contrast, RPS4 was not detected in the DSC2_YFP precipitates, although a faint RPS4 band was observed in the YFP_DSC2 precipitates when expressed at similar levels to DSC1 at 24 hpi (Figure 7E). In line with this, expression of RPS4 alone triggered strong and rapid HR-like cell death only a few days after infiltration which was not suppressed by co-expression with CAMTA3 (Figure 7F). These data show that DSC2 preferentially co-immunoprecipitates with DSC1, indicating that they may be found in complexes in planta.

Discussion

Arabidopsis autoimmune mutants have been intensively studied for more than 20 years (Dietrich et al., 1994; Greenberg and Ausubel, 1993; Greenberg et al., 1994). Many reports link autoimmune phenotypes to NLR signaling pathways, and recently also directly to NLR genes (Bonardi et al., 2011; Bruggeman et al., 2015; Palma et al., 2010; Roux et al., 2015; Zhang et al., 2012). We hypothesized that numerous phenotypes related to autoimmunity may be caused by NLR activation. This implies that specific mutations in host plant guardees mimic pathogen effector activities and trigger the corresponding NLR guards.

To examine this possibility we introduced specific mutations into the P-loops of a large collection of NLRs. This approach was supported by i. the conservation of the P-loop in the STAND and closely related NLR families (Leipe et al., 2004), ii. the dominant negative effects of such mutations on the Arabidopsis LAZ5 and tobacco N NLRs (Dinesh-Kumar et al., 2000; Mestre and Baulcombe, 2006; Palma et al., 2010), and iii. the possibility that numerous NLRs form dimers or directly or indirectly associate in complexes (Mestre and Baulcombe, 2006) leading to dominant negative subunit poisoning.

As proof-of-concept, we compromised the function of RPM1 by expressing its dominant negative version, and showed that this suppression was specific and did not affect general NLR function (Figure 1 and 2). Abolition of RPM1 function by P-loop mutation was shown
previously (Chung et al., 2011; Tornero et al., 2002), but not the dominant negative effect described here. In addition, we found that expression of \textit{SUMM2-DN} suppressed autoimmunity in \textit{pat1} mutants (Figure S1). Thus, \textit{NLR-DN} alleles can be made via simple P-loop mutagenesis.

We then conducted a screen to identify other \textit{NLR-DN} alleles which suppress the autoimmune phenotype of the \textit{camta3} mutant (Galon et al., 2008). This screen identified \textit{DSC1-DN} and \textit{DSC2-DN}. CAMTA3 was described as a negative regulator of immunity due to the ectopic accumulation of defense related transcripts including \textit{EDS1} in \textit{camta3} mutants, and to the suppression of \textit{camta3} phenotypes in \textit{camta3 eds1} double mutants (Du et al., 2009). However, \textit{EDS1} is ectopically expressed in many autoimmune mutants, and \textit{EDS1} mutations suppress autoimmunity in many of them (Bruggeman et al., 2015; Rodriguez et al., 2016). We show here that \textit{EDS1} mRNA levels are similar to those in wild type when \textit{DSC1-DN} or \textit{DSC2-DN} is expressed in \textit{camta3} (Figure 3E & 6D). Importantly, the increased resistance to virulent \textit{Pst DC3000} in \textit{camta3} was also reduced to WT levels in \textit{camta3} expressing either \textit{DSC1-DN} or \textit{DSC2-DN} (Figure 3C & 6B). These findings do not support a function for CAMTA3 as a negative regulator of either \textit{EDS1} expression or of resistance to the pathogen tested.

Nonetheless, Du et al. (2009) provided other evidence that CAMTA3 negatively regulates \textit{EDS1} expression. First, CAMTA3 recognized an \textit{EDS1} promoter element which was responsible for suppression of a reporter gene driven by the \textit{EDS1} promoter. Second, chromatin-immunoprecipitation in protoplasts with transiently over-expressed, YFP-tagged CAMTA3 showed enrichment of \textit{EDS1} promoter elements, confirming that CAMTA3 can bind \textit{EDS1} promoter elements (Du et al., 2009). Third, plants which overexpress \textit{CAMTA3} exhibit increased susceptibility to virulent \textit{Pst DC3000} (Jing et al., 2011). Nonetheless, these and other data (Du et al., 2009; Nie et al., 2012) do not provide direct evidence for inhibition of transcription by CAMTA3. More recently, the Rapid Stress Response Element (RSRE), characterized in promoters that rapidly respond to stresses including flg22 (Walley et al., 2007), was identified as a core CAMTA3 binding element. In addition, CAMTA3 could transiently activate the expression of a RSRE:LUC reporter (Benn et al., 2014), and a \textit{camta3} mutant exhibited reduced RSRE:LUC activity (Bjornson et al., 2014). Similarly, a general stress response and RSRE induction is CAMTA3 dependent (Benn et al., 2016). These findings indicate that CAMTA3 is a positive regulator of early stress responses. While CAMTA3 may thus possess both positive and negative regulatory activities, our data indicate that autoimmunity in \textit{camta3} is NLR triggered.
Our results are consistent with a model in which DSC1 and DSC2 guard CAMTA3 and/or a complex or pathway in which CAMTA3 functions. Importantly, we show that CAMTA3 may exist in complexes with DSC1 in planta (Figure 4B-D). Moreover, both DSC1 and DSC2 can trigger the HR when expressed in *N. benthamiana*, but co-expression of CAMTA3 prevents this (Figure 4A and 6E). Thus, these two NLRs appear inactive in the presence of CAMTA3 but are activated in its absence. This is analogous to immunity triggered by RPS2 upon effector-mediated degradation of the host guardee RIN4 (Axtell and Staskawicz, 2003; Mackey et al., 2003).

Interestingly, *camta3* autoimmune phenotypes were not suppressed in *camta3 dsc1* or *camta3 dsc2* double loss-of-function mutants. This may be explained if both DSC1 and DSC2 can be triggered in the absence of CAMTA3. In line with this, autoimmunity was completely suppressed in *camta3 dsc1 dsc2* triple loss-of-function mutants. Thus, autoimmunity in *camta3* can be triggered by both NLRs, and the function of both must be abrogated to prevent autoimmunity.

These findings and the co-immunoprecipitation of DSC1 with DSC2 in *N. benthamiana* suggest that they interact. Such interactions probably do not only involve direct heterodimerization under natural conditions, as such heterodimer formation would be disrupted in their single *dsc1* or *dsc2* loss-of-function mutants leading to suppression of *camta3* mutant phenotypes. Alternatively, the DSC1 and DSC2 co-precipitation is consistent with indirect associations via complexes with CAMTA3 or an *N. benthamiana* orthologue. In a simple model, if activation of such complexes was dependent on either or both DSC1 and/or DSC2, then activation might be compromised by over-expression of the DN form of either NLR but not by loss-of-function of either single NLR. Elucidation of such models requires further biochemical and structural work on plant NLR self-association interfaces (Zhang et al., 2017) in light of animal NLR oligomerization (Hu et al., 2015).

More than one NLR may contribute to autoimmunity in other mutants including *acd11* and *mpk4*. For example, *laz5-D2* can fully suppresses *acd11* autoimmunity although *acd11 laz5-1* doubly homozygous recessive mutants still display significant cell death and activated defense under certain growth conditions (Palma et al., 2010). Similarly, *summ2* only partially suppresses *mpk4* (Zhang et al., 2012). In addition, some NLRs function in pairs of a sensor and a trigger (Cesari et al., 2014; Roux et al., 2015; Sarris and Jones, 2015). However, since both DSC1 and DSC2 can independently trigger immunity in the absence of CAMTA3, it is unlikely they constitute a sensor/trigger pair. It appears more likely that different activities of microbial effectors targeting CAMTA3, or CAMTA3 containing complexes or pathways, are differentially sensed by...
DSC1 and 2. According to the NLR phylogeny of Meyer et al. (2003) DSC1 and DSC2 are not especially closely related. This may not be surprising however, as two NLRs which monitor RIN4 function, RPS2 and RPM1, are also not especially closely related.

We conclude that our screen to link NLR-DN alleles to potential guardees is a more robust and timely method than suppression screens of double loss-of-function mutants. Furthermore, our dominant negative method can identify NLRs with redundant functions or working in pairs. If exploited, our collection of NLR-DN constructs (Table S1) should clarify the relationships between guardees and numerous ‘negative regulators’ of immunity and cell death in plants.

STAR Methods

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Morten Petersen (shutko@bio.ku.dk).

Experimental Model and Subject Details

**Arabidopsis thaliana**

*Arabidopsis* plants were grown in 9 x 9 cm pots in growth chambers at 22°C and ~70% relative humidity and with an 8-hour photoperiod. The intensity of the light was set at ~140 μE m⁻² s⁻¹. The following *Arabidopsis* lines were used in this study: wild type Colombia (Col-0), camta3-1 (referred to as camta3), SALK_001152 (Galon et al., 2008); dsc1, Sail_49_C05; dsc2, SALK_009668; rpm1-3 (Grant et al., 1995); pat1-1, summ2-8 (Zhang et al., 2012); pat1/summ2 (Roux et al., 2015); ndr1-1 (Century et al., 1995) and eds1-2 (Parker et al., 1996; Aarts et al., 1998). All lines have been authenticated by genotyping; the primers used are listed in Table S2. All P-loop mutated NLR lines created in this study is listed in Table S1.

**Nicotiana benthamiana**

Plants were grown in greenhouses under controlled conditions (24°C and 40-65% relative humidity), and a long-day photoperiod (16 hour light and 8 hour dark). Illumination were set to ~130-150 μE m⁻² sec⁻¹.
*Escherichia coli*

E.coli (XL blue) were grown on LB plates with appropriate antibiotic at 37°C and kept at 4°C for up to two weeks. For liquid cultures a bacterial scrape were inoculated in 5 ml LB supplemented with appropriate antibiotics and grown at 37°C under shaking.

*Agrobacterium tumefaciens*

Strains of Agrobacterium (GV3101 and Agl-1) were grown on YEP plates with appropriate antibiotic at 28°C and kept at 4°C for up to two weeks. For liquid cultures a bacterial scrape were inoculated in 5 ml YEP supplemented with appropriate antibiotics and grown at 28°C under shaking. After 24 hours, YEP was added to a total volume of 11 ml.

*Pseudomonas syringae pv. tomato DC3000*

Pst. DC3000 strains were grown on NYG plants containing 100 µg/ml rifampicin, 12.5 ng/ml kanamycin, and 50ng/ml cyclohexamide at 28°C for two days. For liquid cultures 5 ml NYC supplemented with kanamycin and rifampicin were inoculated with a slab of bacteria. *Pst.* DC3000 containing the avirulence genes avrRpm1 (Grant et al., 1995), avrRps4 (Hinsch and Staskawicz, 1996), avrRpt2 (Bent et al., 1994) in the broad host range vector pVSP61, or DC3000 containing empty pVSP6, were used in this study.

**Method details**

**Cloning**

WT CAMTA3, DSC1 and DSC2 was amplified from genomic DNA (from Col-0 plants) without STOP codon and cloned into a modified USER compatible pENTR vector using uracil-excision based cloning (USER, New England Biolabs). Cloning primers were tagged with 5’-ggettaaU3’ for the forward primer and 5’-ggtttaaU3’ for the reverse primer. Constructs were transferred to Gateway-compatible constitutive expression vectors by LR recombination reaction (Invitrogen). Plasmids were verified by sequencing and then electrophoresed into *Agrobacterium tumefaciens* GV3101.

For subcellular localization, FRET, cell death and immunoprecipitation, CAMTA3 were transferred to pGWB645 (35S pro, N-terminal CFP) and pGWB514 (35S pro, C-terminal HA); DSC1 were
transferred to pGWB542 (35S pro, N-terminal YFP) and pGWB515 (35S pro, N-terminal HA) and DSC2 were transferred to pGWB541 (35S pro, C-terminal YFP) and pGWB542.

GFP was PCR amplified from plasmid template and cloned into pENTR/D-TOPO (Invitrogen). The construct was subsequently transferred to the Gateway-compatible constitutive expression vector pGWB517 (35S pro, C-terminal MYC) by LR recombination reaction (Invitrogen). The plasmid were verified by sequencing and then electrophoresed into Agrobacterium tumefaciens Agl-1.

CAMTA1 and CAMTA2 in pENTR/D-TOPO were obtained from the ABRC Stock center. CAMTA1 and CAMTA2 were transferred to pGWB645 (35S pro, N-terminal CFP) by LR recombination reaction (Invitrogen). Plasmids were verified by sequencing and then electrophoresed into Agrobacterium tumefaciens GV3101.

Generation of transgenic Arabidopsis lines

To generate the double mutants, camta3-1 homozygous plants were crossed with homozygous dsc1 or dsc2. Homozygous double mutant plants were identified in the F2 progeny by PCR. For the camta3/dsc1/dsc2 triple mutant, homozygous camta3/dsc1 double mutants were crossed with homozygous camta3/dsc2. Homozygous triple mutants were identified in the F2 progeny by PCR. Homozygosity and correct insertion T-DNA sites were verified by PCR using standard conditions. Genotyping primers for T-DNA lines are provided in Table S2.

Generation of camta1 camta3 DSC1-DN lines was done by genetic crossing of homozygote lines of the camta1 camta3 double mutants and DSC1-DN. Homozygous triple mutants were identified by PCR. Homozygosity and correct insertion T-DNA sites were verified by PCR using standard conditions. Genomic constructs used to complement camta3 dsc1 dsc2 triple mutants was inserted in pGWB601. Plants were transformed by floral dip (Clough and Bent, 1998).

NLR P-loop collection

P-loop mutated NLRs were created from genomic DNA by USER mutagenesis (Nour-Eldin et al., 2006) and cloned into a modified USER compatible pCAMBIA-3300, using uracil-excision based cloning (USER, New England Biolabs). Cloning primers were tagged with 5'-ggtttaaU3' for the forward primer and 5'-ggtttaaU3' for the reverse primer. Mutagenesis primers were made containing the p-loop mutation GXXXXGKT(T/S) to GXXXXAAT(T/S) of the P-loop motif and appropriate
uracil’s incorporated to give seamless overlap of two fragments (Geu-Flores et al., 2007) generated with PfuX7 (Norholm, 2010).

The final constructs were verified by sequencing, electrophoresed into Agrobacterium tumefaciens strain GV3101 and used to transform camta3 or wild type plants by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on soil with BASTA (120 mg/ml).

**Ion leakage**

Four-week-old plants were syringe inoculated with *Pst. DC3000* (avrRpm1) at OD$_{600}$ = 0.2. Four leaf discs were punched out. Samples were taken from one side of the leaf between the central vein and leaf margin. Leaf discs were washed in distilled H$_2$O for to eliminate signal derived from wounded cells. Four discs from each line were then placed in tubes containing fresh distilled H$_2$O, and measurements of solution conductivity were taken at the indicated time points using a conductivity meter.

**Resistance assay**

For bacterial growth assays, leaves of 5-week-old soil grown plants were inoculated by syringe infiltration (OD$_{600}$ = 0.001) with *Pst* DC3000 either containing avirulence genes or the empty vector. Bacterial growth (Colony forming units per cm$^2$) was determined 3 days post inoculation, day 0 counts were analyzed in infiltrated leaves to ensure that no statistical difference was present at inoculation and that day 3 showed positive growth. The experiments were repeated in at least three individual biological replicates, each with three technical replicates.

**Trypan blue staining**

Leaves of 6 week-old plants were boiled in Trypan blue 2-3 min and destained in chloral hydrate. Leaves were placed on slides in 50% glycerol for visualization of dead cells.

**Quantitative Real-Time PCR**

RNA was extracted from plant leaves using the NucleoSpin® RNA Plant kit (Machery-Nagel). First-strand cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit according to the manufacturer’s instructions (Thermo Scientific). The constitutively expressed *UBQ10* gene was used as an internal control. qRT-PCR analysis was performed on a Bio-RAD
CFX96 system with the dye SYBR Green (Thermo Scientific). All experiments were repeated at least three times each in technical triplicates. Primer sequences are listed in Table S2.

**Cell death**

For transient expression *N. benthamiana* was syringe infiltrated with *Agrobacterium* at OD$_{600} =$ 0.5 expressing indicated constructs. GV3101 carrying 35S p19 was co-infiltrated at OD$_{600} =$ 0.2. For cell death assays leaves were analyzed ~3 dpi.

**Subcellular localization and FRET**

*N. benthamiana* was infiltrated with *Agrobacterium* at OD$_{600} =$0.5 expressing indicated constructs. For subcellular localization and FRET, leaf disks were analyzed 2 or 3 dpi. Subcellular localization was done using a LSM700 Zeiss confocal microscope. All samples were imaged with a 63X water objective. The confocal images were edited with Zen2012 (Zeiss) software. FRET-AB was done using a Leica SP5-X inverted confocal microscope. All experiments were done with a 63X water objective. FRET analysis was performed using Leica FRET-AB wizard software.

**Protein extraction and Co-immunoprecipitation**

*N. benthamiana* was infiltrated with *Agrobacterium* at OD$_{600} =$0.5 expressing indicated constructs. GV3101 carrying 35S p19 was co-infiltrated at OD$_{600} =$ 0.2. Protein were extracted 24 hpi in 50mM Tris-HCl pH 7.5; 150mM NaCl; 10% (v/v) glycerol; 10mM DTT; 10mM EDTA; 0.5% (v/v) PVP; protease inhibitor cocktail (Roche); 0.1% (v/v) Triton X-100 added at 2ml/g tissue powder. Following 20 min centrifugation at 4°C and 13000 rpm sample supernatants were adjusted to ~3mg/ml protein and incubated 2 hours at 4°C with GFPTrap-A beads (Chromotek) or anti-HA antibody (Santa cruz) and EZview protein A agarose beads (Sigma). Beads were washed [20mM Tris pH 7.5; 150mM NaCl; 1mM EDTA] before adding 2x SDS and heating at 80°C.

**SDS-PAGE and immunoblotting**

Protein samples were separated on 8% SDS-PAGE, electroblotted to PVDF membrane (GE Healthcare), then blocked (1 hour in 5% (w/v) BSA or 5% (w/v) milk in TBS-Tween (0.1%)) and incubated 2 hours to overnight with primary antibodies: anti-GFP 1:5000 (AMS Biotechnology), anti-HA 1:1000 (Santa cruz), anti-HA 1:1000 (Cell Signaling). Membranes were incubated in secondary antibodies, anti-rabbit or anti-mouse AP or HRP conjugate (Promega; 1:5000) for 1 hour.
Chemiluminescent substrate (homemade or ECL Plus, Pierce) was applied before exposure to film (AGFA CP-BU) or camera detection. For AP-conjugated antibodies, membranes were incubated in NBT/BCIP (Roche) until bands were visible.

**Quantification and statistical analysis**

Statistical details of experiments are reported in the figures and figure legends. In short, n = 3 for all samples if nothing else is stated and +/- standard deviation of the mean is indicated by error bars. Means not sharing the same letter are significantly different. Statistical significance between groups was determined by ANOVA One-Way comparison followed by Tukey’s HSD (honest significant difference) test, P < 0.05, was used unless otherwise stated. At least three individual replicas were always included. All statistics were done using the software OriginPro (OriginLab).
Author contributions
S.L., C.G., K.P., M.R., M.K.J., S.B., E.E. and K.S. performed the experiments; S.L., C.G., J.M. and M.P. designed the experiments; S.L., C.G., J.M. and M.P. wrote the manuscript.

Conflict of interest

Acknowledgements
We thank Suksawad Vonvisuttikun for technical help. We thank Tsuyoshi Nakagawa (Shimane University) for providing Gateway binary pGWB vectors. RPS4_HA were kindly provided by Jonathan D. G. Jones (The Sainsbury Laboratory). The camta1 camta3 double line was a gift from Michael F. Thomashow (Michigan State University). All confocal work was done at Center for Advanced Bioimaging.

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References


Belkhadir, Y., Nimchuk, Z., Hubert, D.A., Mackey, D., and Dangl, J.L. (2004). Arabidopsis RIN4 negatively regulates disease resistance mediated by RPS2 and RPM1 downstream or independent of the NDR1 signal modulator and is not required for the virulence functions of bacterial type III effectors AvrRpt2 or AvrRpm1. The Plant cell 16, 2822-2835.


Figure 1. Expression of RPM1-DN compromises RPM1 function and attenuates resistance. (A) AvrRpm1 dependent cell death is inhibited by RPM1-DN expression. Ion leakage assay after inoculation of Pst DC3000 (AvrRpm1) into Col-0, rpm1-3, or two transgenic lines overexpressing RPM1-DN (line RPM1-DN1 and 2). Error-bars represent +/- SD. Groups with statistically different means are indicated by different letters. (B and C) Col-0 resistance to Pst DC3000 (AvrRpm1) is compromised by RPM1-DN expression. No effect is seen in resistance to Pst DC3000 carrying an empty vector. Growth of Pst DC3000 (AvrRpm1 or empty vector) at day 0 (grey) and 3 (black) as log10-transformed colony-forming units/cm² leaf tissue (cfu/cm²). Error-bars represent +/- SD (n = 4). Means not sharing the same letter are significantly different.
Figure 2. *RPM1-DN* expression specifically inhibits RPM1 function and does not compromise resistance to AvrRps4 or AvrRpt2.

Wild type resistance to *Pst* DC3000 (AvrRps4) and (AvrRpt2) is retained in transgenic *RPM1-DN* plants. Col-0, rpm1-3, RPM1-DN1, RPM1-DN2 and eds1-2 or ndr1-1 were inoculated with *Pst* DC3000 (AvrRps4) (A) and (AvrRpt2) (B). Bacterial growth was measured on day 0 (grey) and 3 (black). Mean +/- SD for n=4 are shown and means not sharing the same letter are significantly different.
Figure 3. DSC1-DN suppresses camta3 autoimmune phenotypes.

(A) camta3 growth and chlorosis phenotypes are rescued by expression of DSC1-DN. Pictures are representative for several individual lines.

(B) camta3 microscopic lesions are suppressed by expression of DSC1-DN. Trypan Blue stained leaves of Col-0, camta3, DSC1-DN and camta3 DSC1-DN. One representative of several individual lines is shown.

(C) Expression of DSC1-DN suppresses resistance in camta3 mutants. Plants were inoculated with Pst DC3000 and cfu/cm² are plotted for days 0 (gray) and 3 (black). Error bars represent +/- SD among four samples.

(D and E) PR1 and EDS1 mRNA levels are elevated in camta3 mutants, but reduced to wild type levels in camta3 expressing DSC1-DN. mRNA levels for PR1 (D) and EDS1 (E). +/- SD of the mean is indicated by errors bars (n = 3). Means not sharing the same letter are significantly different.
Figure 4. CAMTA3 is associated with DSC1 in *N. benthamiana*

(A) Expression of CAMTA3 rescues DSC1 induced cell death in *N. benthamiana*. Inoculation with *Agrobacterium* expressing DSC1 resulted in HR induction. Co-inoculation with CAMTA3 inhibited this DSC1 induced cell death. Co-inoculation with CAMTA1 or CAMTA2 failed to inhibit the induction of HR. Dashed lines mark infiltrated areas.

(B and C) Detection of *in vivo* interaction between CAMTA3 and DSC1 by FRET in *N. benthamiana*. CFP_CAMTA3 and YFP_DSC1 were co-expressed in *N. benthamiana* and analyzed for FRET-AB. CFP-MPK4 and YFP-PAT1 were included as positive controls. Negative controls are CFP_CAMTA with YFP_SUMM2 and CFP_MPK4 with YFP_DSC1. Error bars represent +/- SD among four samples. Means not sharing the same letter are significantly different. See also Figure S3.

(D) CAMTA3 is associated with DSC1 in *N. benthamiana*. HA_DSC1 + CAMTA3_CFP or GFP_MYC were co-expressed in *N. benthamiana* and tissue harvested 24 hpi. Immunoblots of input and anti-HA IPs probed with anti-HA and anti-GFP antibodies. Left panel, anti-HA IP; right panel, input. Black arrow: HA_DSC1; grey arrow: CAMTA3_CFP; white arrow: GFP_MYC.
Figure 5. A T-DNA insertion in DSC1 does not suppress the camta3 autoimmune phenotypes.

(A) The phenotypes of camta3 and camta3 dsc1 are indistinguishable. Morphological phenotypes of Col-0, camta3, dsc1, and camta3 dsc1 double mutant.

(B) Resistance is similar in camta3 dsc1 double and camta3 mutants. Plants were infiltrated with Pst DC3000 and cfu/cm2 plotted for days 0 (gray bars) and 3 (black). Error bars represent +/- SD, n=4.

(C and D) PR1 and EDS1 mRNA levels in the camta3 dsc1 double mutant are the same as in camta3. Relative PR1 (C) and EDS1 (D) mRNA levels in camta3, dsc1 and camta3 dsc1 mutants determined by RT-qPCR. Error bars represent +/- SD of n=3. Bars with different letters are statistical significantly different.
Figure 6. DSC2-DN transgene suppresses the camta3 autoimmune phenotypes.

(A) camta3 growth and chlorosis phenotypes are rescued by expression of DSC2-DN. Pictures are representative for several individual lines.

(B) Expression of DSC2-DN suppresses resistance in camta3 mutants. Plants were syringe inoculated with Pst DC3000 and cfu/cm2 counted on days 0 (gray bars) and 3 (black). Error bars represent +/- SD, n=4. Bars with different letters are statistically significantly different.

(C) PR1 mRNA levels are reduced to almost wild type levels in camta3 mutants expressing DSC2-DN. PR1 mRNA expression levels. Error bars represent +/- SD, n=3. Letters indicate statistical significance.

(D) EDS1 mRNA levels are elevated in camta3 mutants, but are similar to Col-0 levels in camta3 DSC2-DN. mRNA levels for EDS1. Standard deviation of the mean is shown by errors bars (n = 3). Means not sharing the same letter are significantly different.

(E) Expression of CAMTA3 rescues DSC2 induced cell death in N. benthamiana. Inoculation with Agrobacterium expressing DSC2 results in induction of HR. Co-inoculation with CAMTA3 inhibits the DSC2 induced cell death. Co-inoculation with CAMTA1 or CAMTA2 failed to inhibit HR induction. Areas of infiltration are marked by dashed lines.
Figure 7. *camta3 dsc1 dsc2* triple mutants rescue all *camta3* phenotypes.

(A) *camta3 dsc1 dsc2* triple mutants develop like wild type Col-0. Representative images of several individual lines are shown. See also Figure 5, S4 and S5.

(B) *camta3* resistance to *Pst DC3000* is suppressed in the triple *camta3 dsc1 dsc2* mutant. cfu/cm2 were counted at day 0 (grey bars) and 3 (black). +/- SD of the mean is indicated by errors bars (n = 4). Bars with different letters are significantly different.

(C and D) *PR1* and *EDS1* mRNA levels in *camta3* are completely abolished in the *camta3 dsc1 dsc2* triple mutant. mRNA levels for *PR1* (C) and *EDS1* (D) are shown relative to wild type Col-0. +/- SD of the mean is indicated by errors bars (n = 3). Means not sharing the same letter are significantly different.

(E) DSC2 is associated with DSC1 in *N. benthamiana.*

*DSC1_HA + DSC2_YFP, YFP_DSC2* or *GFP_MYC* and *RPS4_HA + DSC2_YFP* or *YFP_DSC2* were co-expressed in *N. benthamiana.* Left panel, anti-HA IP; right panel, input. Black arrow: HA_DSC1/RPS4_HA; grey arrow: DSC2_YFP/YFP_DSC2; white arrow: GFP_MYC.

(F) RPS4 induced cell death in *N. benthamiana* is not rescued by expression of *CAMTA3.* Inoculation of *N. benthamiana* leaves with RPS4 resulted in HR induction. Co-inoculation with CAMTA3 did not affect RPS4 induced cell death. Areas of infiltration are marked by dashed lines.
Expression of *SUMM2-DN* suppresses the autoimmune phenotype of *pat1* mutants. Related to Figure 2.

(A) Expression of *SUMM2-DN* in *pat1* suppresses dwarfism. Morphology of Col-0, *pat1-1, pat1 SUMM2-DN*, and the *pat1 summ2* double mutant. Plants were grown on soil for 5 weeks under short day conditions.

(B) Expression of *SUMM2-DN* in *pat1* suppresses bacterial resistance to the same level seen in *pat1 summ2* double mutants. Leaves of 5 week-old plants were syringe inoculated with *Pst* DC3000. Bacterial counts were conducted 0 days and 3 days post inoculation. Bars represent mean of four replicas, with error bars showing standard deviation. Significant differences between the lines are indicated with lower case letters for *P* < 0.05 determined by ANOVA followed by Fisher’s LSD test.

(C) Similarly to *pat1 summ2* plants, expression of *SUMM2-DN* in *pat1* suppresses elevated levels of *PR1*. *PR1* mRNA levels were determined by RT-qPCR. Data were normalized to UBQ10 and are presented as relative to wild type Col-0 levels. Error bars represent standard deviations (*n* = 3). Significant differences are indicated by lower case lettering, determined by ANOVA, *P* < 0.05. The experiment was performed with triplicate biological samples, each with technical triplicates.
Figure S2

DSC1-DN suppression is specific for camta3 and no rescue is seen when DSC1-DN is expressed in camta1 camta3 double mutants. Related to Figure 3.

(A and B) Morphology of Col-0 wild type, camta3 and camta1 camta3 double mutants is shown in (A). DSC1-DN expressed in Col-0, camta3 and camta1 camta3 background in (B). Plants were grown on soil for 6 weeks under short day conditions.
Figure S3
CAMTA3 and DSC1 have the same subcellular localization in *N. benthamiana* epidermal cells. Related to Figure 4.

(A) Confocal microscopy with *N. benthamiana* leaves 3 days post inoculation with *Agrobacterium* expressing *CFP_CAMTA3*. Clear CFP signal is seen in both nuclei and cytoplasm. Scale bars correspond to 10 µm.

(B) Confocal microscopy *YFP_DSC1* 3 days post inoculation in *N. benthamiana* leaves. Clear YFP signal is seen in both nuclei and cytoplasm. Scale bars correspond to 10 µm.
Figure S4
A T-DNA insertion in DSC2 only partially suppresses the camta3 autoimmune phenotypes. Related to Figure 6 and Figure 7.

(A) The camta3 dsc2 double mutant no longer exhibits dwarfism, but otherwise develops like camta3. Morphological phenotype of WT (Col-0), camta3, dsc2, and camta3 dsc2 double mutant. Plants were photographed 6 weeks after germination.

(B) camta3 dsc2 double mutant’s exhibit induced resistance against bacterial growth. 5 week-old plants were infiltrated with *Pst* DC3000 and the number of bacteria per area of leaf are plotted for day 0 and day 3. Error bars represent the standard deviation among four samples and this experiment is representative of three independent replicates. Statistical significance between the raw data was determined by ANOVA followed by Fisher’s LSD test, \( P < 0.05 \).

(C) PR1 levels in the camta3 dsc2 double mutant are significantly higher than in wild type Col-0 plants. Relative expression of PR1 in 4 week-old Col-0, camta3, dsc2 and camta3 dsc2 mutants. Total RNAs were extracted and submitted to RT-qPCR analysis. Data is normalized to UBQ10 and presented as relative expression compared to Col-0. Error bars represent standard deviation of \( n = 3 \). Bars with different letters are statistical significantly different, determined by ANOVA, \( P < 0.05 \). The experiment was performed with triplicate biological samples, each with technical triplicates.

(D) EDS1 mRNA levels are significantly higher in dsc2 and camta dsc2 double mutants than in wild type Col-0. mRNA levels for EDS1 are shown relative to Col-0 plants as determined by RT-qPCR using the UBQ10 gene as an internal standard. Standard deviation of the mean is indicated by error bars (\( n = 3 \)). Means not sharing the same letter are significantly different, determined by ANOVA followed by Fisher’s LSD test, \( P < 0.05 \). The experiment was performed with triplicate biological samples, each with technical triplicates.
Figure S5

Genomic clones of DSC1 or DSC2 in the camta3 dsc1 dsc2 triple mutant revert the phenotypic suppression. Related to Figure 7.

camta3 dsc1 dsc2 triple mutants complemented with DSC1 or DSC2 resembles camta3 dsc1 and camta3 dsc2 double mutants respectively. Plants with the indicated genotypes were grown on soil for 4 weeks in short day conditions.
### TIR-NB-LRR

| At1g17600 | At1g72840 | At3g44480 | At4g19510 | At5g22690 | At5g41550 | At5g46450 |
| At1g17610 | At1g72870 | At3g44630 | At4g19530 | At5g36930 | At5g41740 | At5g46470 |
| At1g27170 | At1g72900 | At3g51560 | At4g23440 | At5g38340 | At5g41750 | At5g46510 |
| At1g31540 | At1g72910 | At3g51570 | At4g36150 | At5g38350 | At5g44510 | At5g48770 |
| At1g56520 | At1g72940 | At4g09360 | At5g12250 | At5g38850 | At5g45060 | At5g49140 |
| At1g63730 | At1g72950 | At4g12010 | At5g17680 | At5g40060 | At5g45200 | At5g51630 |
| At1g63740 | At2g16870 | At4g16890 | At5g17880 | At5g40090 | At5g45230 | At5g58120 |
| At1g63750 | At2g17050 | At4g16940 | At5g17970 | At5g40100 | At5g45240 |
| At1g63870 | At3g04210 | At4g16950 | At5g18350 | At5g40910 | At5g45260 |
| At1g66090 | At3g04220 | At4g16960 | At5g18360 | At5g40920 | At5g46260 |
| At1g69550 | At3g44400 | At4g19500 | At5g18370 | At5g41540 | At5g46270 |

### CC-NB-LRR

| At1g12220 | At1g15890 | At1g58390 | At1g59620 | At3g14470 | At4g14610 | At5g04720 |
| At1g10920 | At1g33560 | At1g58410 | At1g61180 | At3g15700 | At4g19060 | At5g05400 |
| At1g12210 | At1g50180 | At1g58807 | At1g63360 | At3g46530 | At4g27190 | At5g66630 |
| At1g12280 | At1g52660 | At1g59124 | At3g07040 | At3g50950 | At4g27220 | At5g66900 |
| At1g12290 | At1g53350 | At1g59218 | At3g14460 | At4g10780 | At4g33300 | At5g66910 |

### Table S1

Table listing all dominant negative NLRs produced as part of this study. Related to Figure 3 and Figure 6.
Table S2

Primers used in this study. Related to STAR Methods.

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Introduction to FAPP2

A classic hallmark of plant autoimmunity is the development of spontaneous lesions resembling HR cell death (Bruggeman et al., 2015). Work on plant autoimmune mutants has linked sphingolipid metabolism to establishment of the HR. At least two autoimmune mutants are known to be involved in sphingolipid metabolism, namely $acd5$ and $acd11$ (Brodersen et al., 2002; Bruggeman et al., 2015; Greenberg et al., 2000). Sphingolipids and their metabolites (including ceramide and sphingosine-1-phosphate) are bioactive lipids that function as messenger signals and mediators of eukaryotic processes (Simanshu et al., 2014). Sphingolipids are extensively involved in cell growth, development, embryogenesis, senescence, inflammation and programmed cell death associated with defense (Simanshu et al., 2014). It seems that the balance between ceramides/sphingolipids and their phosphorylated derivatives is important for modulating cell death in plants and animals (Berkey et al., 2012; Liang et al., 2003). Furthermore, a role for sphingolipids in signaling and in regulation of membrane trafficking and formation of membrane subdomains during defense response is also suggested (Berkey et al., 2012; Petersen et al., 2009).

$acd5$ mutants show spontaneous cell death late during development, which is associated with ceramide accumulation, autophagy induction, and mitochondrial ROS accumulation (Bi et al., 2014; Liang et al., 2003). In addition, $acd5$ mutant plants exhibit enhanced susceptibility to $P. syringae$ and $B. cinerea$ (Bi et al., 2014; Greenberg et al., 2000).

ACD11 functions as an intermembrane ceramide-1-phosphate transfer protein (Brodersen et al., 2002; Simanshu et al., 2014; Zhai et al., 2017). Structural homology modeling predicts that ACD11 forms a GLTP fold and is a glycolipid transfer protein (GLTP) superfamily member (Petersen et al., 2008). However, ACD11 is unable to transport glycolipids. $acd11$ disruption dramatically alters the in vivo balance of sphingolipid mediators that regulate eukaryotic-programmed cell death (Simanshu et al., 2014).

Knock-out of $acd11$ also triggers immunity and cell death via the NLR LAZ5 (Palma et al., 2010). HR-like cell death in $acd11$ further requires SA, and is suppressed by expression of a NahG, or by mutations in the $EDS1/PAD4$ genes (Brodersen et al., 2002; Feys et al., 2001). ACD11 shares homology with other sphingolipid transfer proteins such as $Podospora anserina$ HET-C , human GLTP, and human FAPP2 (Brodersen et al., 2002; D'Angelo et al., 2007; Mattjus et al., 2003).
HET-C is implicated in incompatible cell death that is accompanied by, but not dependent upon, autophagy induction (Petersen et al., 2008). Autophagy is a degradation process that mediates non-selective, bulk degradation of cytosolic components as well as selective clearance of toxic or redundant structures in eukaryotic cells (Levine and Klionsky, 2004). Autophagy is also known to maintain cellular homeostasis in response to various stresses including recycling of nutrients upon starvation (Mizushima, 2007; Russell et al., 2014). Deletion of ACD11 triggers immunity-related autophagy and cell death in Arabidopsis (Mattjus et al., 2003; Munch et al., 2015; Palma et al., 2010). However, FAPP2 has not previously been implicated in processes related to autophagy.

FAPP2 is a cytosolic protein and has been proposed to be involved in Golgi-to-cell surface membrane traffic. Binding of phosphatidylinositol 4-phosphate (PtdIns(4)P) through its N-terminal pleckstrin homology (PH) domain allows FAPP2 to localize to the trans-Golgi network (TGN) (D'Angelo et al., 2012; Godi et al., 2004; Vieira et al., 2005). FAPP2 also appears to control the synthesis of complex sphingolipids transported through the Golgi (D'Angelo et al., 2007; D'Angelo et al., 2013). Furthermore, FAPP2 is a dimeric protein that has the capability to form tubules from membrane sheets, an action that is dependent on the (PtdIns(4)P)-binding activity of the PH domain of FAPP2. FAPP2 exerts its membrane tubulating activity by binding the small GTPase Arf1 to induce membrane deformations leading to tubulation at the TGN (Cao et al., 2009). It is further proposed that FAPP2 functions as a sensor for regulating glycolipid levels in the cell.

Although FAPP2 had not been implicated in autophagy or cell death, its homology to ACD11 and the discovery that transgenic expression of GLTP in Arabidopsis acd11 null mutants rescues the autophagy and cell death phenotypes (Petersen et al., 2008) prompted us to consider whether FAPP2 and ACD11 have common functions in plant and animal cells.

Interestingly, we found that removal of FAPP2 leads to increased autophagic activity. As previously reported, we also found that FAPP2 removal leads to decreased transport from the Golgi to the plasma membrane. However, this transport can be re-established if the autophagic machinery is abrogated together with FAPP2 removal. This work is described in further detail in the draft manuscript "Inhibition of autophagy prior to FAPP2 removal restores cargo transfer to the plasma membrane". Since we associate FAPP2 to the molecular machinery of autophagy, it will be interesting to examine if the genes/ mechanisms linking ACD11 and FAPP2 to autophagy are conserved. Future research may resolve whether autophagy induction by FAPP2 depletion involves NLRs. To this end it could be tested if autophagy, a hallmark of induced immunity, can be
suppressed by knock-down NLRs of other immune components not directly involved in autophagy. Furthermore, it could be interesting to test if altered FAPP2 levels affect immune responses to a collection of human pathogens. This may identify FAPP2 as a prototypic, guarded host target in mammals and establish its role in infection.

References


Inhibition of autophagy prior to FAPP2 removal restores cargo transfer to the plasma membrane

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Abstract

Autophagy is a regulated, degradative process for recycling dysfunctional or unnecessary cellular components. Human FAPP2 protein was reported to control Golgi-to-cell-surface transport of specific cargo and sphingolipids via its pleckstrin homology and sphingolipid transfer domains. Interestingly, the FAPP2 sphingolipid transfer domain is similar to those of HET-C from Podospora anserina and ACD11 from Arabidopsis thaliana, two proteins implicated in autophagy. Here we report that down-regulation of FAPP2 leads to ATG7 and BEC1-dependent accumulation of LC3-positive autophagosomes and increased autophagic flux. Surprisingly, prevention of autophagy before FAPP2 knockdown completely restores cargo transfer to the plasma membrane in different cell types. Thus, the effects of FAPP2 depletion on Golgi-to-surface transport may be indirect and caused by increased autophagic activity. Upon autophagy induction, FAPP2 is recruited to ATG5-positive foci in a process dependent on the conjugation of ATG5 and ATG12. Our results therefore associate FAPP2 to the molecular machinery of autophagy and suggest that FAPP2 may function in the regulation of autophagy.
Introduction

Macroautophagy (hereafter referred to as autophagy) is a degradation process that mediates non-selective, bulk degradation of cytosolic components as well as selective clearance of toxic or redundant structures in eukaryotic cells. Autophagy is also known to maintain cellular homeostasis in response to various stresses including recycling of nutrients upon starvation (Levine and Klionsky, 2004; Mizushima and Klionsky, 2007; Russell et al., 2014). Autophagy is based on the formation of autophagosomes that engulf cytosolic components including entire organelles and fuse with lysosomes forming autolysosomes, in which degradation takes place (Levine and Klionsky, 2004; Mizushima, 2007). The process of autophagy is conserved in yeast, animals and plants, and involves the action of autophagy related genes (ATG) that synthesize and coordinate membrane rearrangements to engulf cellular components for degradation (Levine and Klionsky, 2004; Mari et al., 2011; Mizushima et al., 2011). A core set of ATG genes is present in all eukaryotes. For example, autophagosome initiation requires two ubiquitin-like conjugation systems to produce ATG12-ATG5 and ATG8-phosphatidylethanolamine (ATG8-PE) conjugates (Geng and Klionsky, 2008; Russell et al., 2014). The ATG12-ATG5 conjugate interacts with ATG16 to form a multimeric complex associated on the outer surface of expanding autophagosomes, but dissociates from the membranes upon autophagosome completion (Mizushima, 2007). ATG8-PE conjugation involves the cysteine proteinase ATG4 and the E1-like protein ATG7, and lipidated ATG8 is linked to and translocated with autophagosomes to the vacuole (Geng and Klionsky, 2008). One mammalian ATG8 orthologue is named microtubule-associated protein 1 light chain 3 (LC3) and conversion from soluble to lipid bound ATG8/LC3, and subcellular localization of green fluorescent protein (GFP) fused protein, have been used to monitor autophagy (Kabeya et al., 2000; Klionsky et al., 2016). In addition, the ATG12–ATG5–ATG16 complex seems important for the correct localization and lipidation of ATG8 (Fujita et al., 2008; Geng and Klionsky, 2008).

In addition to being involved in recycling of cellular components when nutrients are limited, autophagy has been reported to play major roles in fundamental cellular processes. These include functions as an alternative cell death pathway induced by innate immunity in both animals and plants (Berry and Baehrecke, 2007; Delgado et al., 2008; Hofius et al., 2009; Levine et al., 2011; Yu et al., 2004) and as a pro-survival pathway during many types of stresses (Bernales et al., 2006; MacVicar,
The phosphatidylinositol-4-phosphate adaptor protein-2 (FAPP2) has been proposed to be involved in Golgi-to-cell surface membrane traffic and binding of phosphatidylinositol 4-phosphate (PtdIns(4)P) through its N-terminal pleckstrin homology (PH) domain allows FAPP2 to localize to the trans-Golgi network (TGN) (D'Angelo et al., 2012; Godi et al., 2004; Vieira et al., 2005). FAPP2 also controls the synthesis and steady state levels of complex sphingolipids, and glucosylceramides transported through the Golgi by FAPP2 are specifically converted to globosides (D'Angelo et al., 2007; D'Angelo et al., 2013).

Autophagy has been implicated in transport and cargo delivery for conventional secretion via the ER and Golgi and also in the unconventional secretion of cytoplasmic proteins without leader sequences. For example, during starvation accompanied with increased autophagic activity, cargo transport from the Golgi to the plasma membrane is attenuated (Shorer et al., 2005) and apolipoprotein-B secretion is prevented upon exit from the Golgi by autophagic degradation (Pan et al., 2008). Furthermore, knockdown of the autophagy genes Atg5 or Atg7 inhibits the \textit{in vitro} secretion of von Willebrand factor (Torisu et al., 2013). While these findings indicate that increased autophagic activity can prevent conventional secretion, autophagy positively contributes to secretion of pro-inflammatory cytokines released via unconventional secretion (Dupont et al., 2011; Lee et al., 2016).

The C-terminus of FAPP2 is responsible for its glycosphingolipid transfer activity and shares homology with other sphingolipid transfer proteins such as human GLTP, \textit{Podospora anserina} HET-C and \textit{Arabidopsis} ACD11 (Brodersen et al., 2002; D'Angelo et al., 2007; Kamlekar et al., 2013; Mattjus et al., 2003; Simanshu et al., 2014). HET-C is implicated in incompatible cell death that is accompanied by, but not dependent upon, autophagy induction, while deletion of \textit{ACD11} triggers immunity and cell death via cytoplasmic immune receptors also known to induce autophagy in plants (Brodersen et al., 2002; Hofius et al., 2009; Mattjus et al., 2003; Munch et al., 2015; Palma et al., 2010). However, FAPP2 has not previously been implicated in processes related to autophagy.

Here we show that removal of FAPP2 leads to increased autophagic activity. As previously reported, we also find that FAPP2 removal leads to decreased transport from the Golgi to the plasma membrane. However, this transport can be re-established if the autophagic machinery is abrogated
together with FAPP2 removal. The proposed function of FAPP2 in secretion may therefore be more indirect than previously proposed.

RESULTS

FAPP2 removal promotes accumulation of LC3-GFP foci

Both heterokaryon incompatibility in *P. anserina* and immunity in *Arabidopsis* are accompanied by autophagy and can be triggered by mutations in *HET-C* and *ACD11* respectively (Hofius et al., 2009; Munch et al., 2015; Paoletti and Clave, 2007). As the HET-C and ACD11 sphingolipid transfer domains are similar to the C-terminal domain of FAPP2 (Brodersen et al., 2002; Kamlekar et al., 2013), we investigated if FAPP2 depletion triggers autophagy in mammals. We first examined the effect of reducing endogenous FAPP2 expression using small interfering RNAs (siRNAs) in HeLa cells in combination with expression of a GFP-tagged version of human LC3. HeLa cells co-transfected with siFAPP2 and GFP-LC3 accumulated significantly higher numbers of LC3 containing foci associated with autophagosomes compared to control cells transfected with GFP-LC3 and control siRNA (Figure 1A and B). Next, we performed immunoblot analysis of proteins extracted from GFP-LC3 cells co-transfected with siFAPP2 and found increased levels of both LC3 and cleaved GFP compared to cells transfected with control siRNAs and GFP-LC3 (Figure 1C). This indicates that autophagy is induced in FAPP2 depleted cells (Mizushima and Levine, 2010).

To substantiate these findings, we conducted the same assays with Jurkat cells. As seen in HeLa cells, Jurkat cells co-transfected with GFP-LC3 and siFAPP2 also accumulated large numbers of GFP-LC3 containing foci compared to control cells (Supplemental Figure 1A). To examine if our siRNA knocked down FAPP2 expression, we measured relative FAPP2 mRNA levels by qPCR. siRNAs against FAPP2 led to a notable decrease in FAPP2 mRNA (Supplemental Figure 1B). Finally, we used transmission electron microscopy (TEM) to detect autophagosomes. In contrast to siSR treated control cells, FAPP2 depleted cells exhibited a considerable increase in the formation of double membrane vesicles considered to be a hallmark of autophagy (Klionsky et al., 2016) (Supplemental Figure 1C). Morphometric analysis of the TEM images showed accumulation of both autophagosomes and
autolysosomes in cells depleted of FAPP2, suggesting an induction of autophagy rather than a blockage in early autophagic stages.

To exclude the possibility that accumulation of GFP-LC3 positive foci was caused by knockdown of other targets, we included siFAPP2v used in previous reports (D'Angelo et al., 2007) as well as siFAPP2b designed by us. In addition, we wanted to complement the effects of FAPP2 knock down with a siRNA-resistant version of FAPP2. To this end, two FLAG-tagged FAPP2 constructs were co-transfected with the siRNAs in HeLa cells. p3XFLAG-FAPP2-WT contains the wild type FAPP2 sequence and should be targeted by both siRNAs, while p3X-FALG-FAPP2-Mutant contains a synonymous FAPP2 sequence mutation that hinders siFAPP2b binding. While accumulation of FLAG-FAPP2-WT protein was effectively abolished in cells co-transfected with either of the siRNAs, accumulation of the mutated version of the FLAG-FAPP2 protein was not affected by the presence of siFAPP2b (Figure 2A). This was supported by the significant accumulation of LC3 positive foci when FLAG-FAPP2-WT was co-expressed with either FAPP2 directed siRNA, whereas cells co-transfected with p3X-FALG-FAPP2-Mutant and siFAPP2b did not accumulate LC3 foci (Figure 2B and C). These results indicate that i. the siFAPP2s used are specific, ii. depletion of FAPP2 leads to induction of autophagy, and iii. this induction is not caused by off target effects of siFAPP2.

**Autophagy induced by FAPP2 knock-down is ATG7 dependent**

Since ATG7 and Beclin-1 (Bec1) are essential for autophagy (Kim et al., 1999; Liang et al., 1999; Tanida et al., 1999), we examined the effects of reducing endogenous ATG7 or Bec1 by siRNAs used in previous reports (Yu et al., 2004) in combination with siFAPP2 treatment. This revealed that GFP-LC3 foci accumulation due to loss of FAPP2 was significantly lowered by ATG7 or Bec1 removal (Figure 3A and B). To support these data, we examined the levels of LC3-II in cells treated with siFAPP2, siATG7 or a combination of both. Only cells transfected with siFAPP2 alone showed enhanced levels of LC3-II (Figure 3C) in keeping with increased levels of autophagic vesicles in these cells. We also included an antibody against FAPP2 to verify that the double knockdown did not affect FAPP2 protein levels (Figure 3C). We then repeated the experiments in Jurkat cells. As expected, knockdown of either AGT7 or Bec1 in combination with FAPP2 depletion completely abolished GFP-LC3 foci accumulation seen in cells only transfected with siFAPP2 (Supplemental Figure 2A and B). These results reveal that removal of FAPP2 leads to increased accumulation of autophagosomes and
that this accumulation depends on two components, ATG7 and Bec1, which are required for functional autophagy.

**Knock-down of FAPP2 results in increased autophagic flux**

Since autophagy can be induced by starvation, we starved cells co-transfected with siRNA and GFP-LC3 to assess the level of autophagy induction by FAPP2 knockdown. In cells transfected with control siSR, starvation using Hank’s balanced salt solution (HBSS) led to increased foci accumulation, whereas in cells depleted for FAPP2 starvation did not affect the average number of foci per cell. This indicates that removal of FAPP2 results in strong induction of autophagy (Figure 4).

The accumulation of GFP-LC3 foci upon FAPP2 removal may reflect either increased autophagic flux, and therefore enhanced formation of autophagosomes, or a block in autophagy resulting in reduced removal of autophagosomes (Klionsky et al., 2016; Mizushima and Levine, 2010; Ni et al., 2011). To discriminate between these two possibilities, we blocked the fusion between autophagosomes and lysosomes with Bafilomycin A1, a widely used inhibitor of the late phase of autophagy which specifically inhibits the vacuolar H+-ATPase (V-ATPase), and quantified LC3 positive foci per cell. HeLa cells co-transfected with GFP-LC3 and siRNA against FAPP2 showed significant accumulation of foci (Figure 4). That blockage of late autophagy steps by Bafilomycin A1 led to accumulation of autophagosomes indicates that depletion of FAPP2 induces autophagy rather than blocking autophagosome removal. Immunoblot analysis of HeLa cells starved after transfection with siFAPP2 also showed no further accumulation of LC3-II (Supplemental figure 3), again supporting robust induction of autophagy upon FAPP2 removal. A slight enhancement in LC3-II accumulation was seen in cells treated with Bafilomycin post transfection with siFAPP2. This confirms the accumulation of autophagosomes upon blockage of their degradation by Bafilomycin (Supplemental Figure 3). Taken together, these results support a model in which depletion of FAPP2 leads to accumulation of LC3 positive autophagosomes due to increased autophagic activity rather than to inhibition or blockage of autophagosome maturation.

**Increased autophagic activity disrupts cargo transfer from Golgi to the plasma membrane**

We and others previously proposed that FAPP2 is involved in Golgi to plasma membrane traffic, and that knockdown of FAPP2 inhibits cargo transfer to the plasma membrane (Godi et al., 2004; Vieira et
al., 2005). It has also been shown that starvation leads to inhibited transport to the plasma membrane in Chinese Hamster cells (Shorer et al., 2005). Since starvation can also induce autophagy which is known to impact multiple cellular functions, we speculated that autophagy induction could alter the efficiency of the secretory pathway. This might then explain the delay in protein to plasma membrane transport seen in FAPP2 deficient cells.

To examine this possibility, we first conducted an assay in HeLa cells similar to that which Godi et al. (2004) used to determine the role of FAPP2 in cargo transfer. We used the temperature sensitive mutant of vesicular stomatitis virus G glycoprotein (VSV-G-ts045) to monitor transport by assessing its arrival at the plasma membrane by immunofluorescence microscopy. Transport was calculated as the ratio of the integrated value of staining for plasma membrane localized VSVG relative to whole cell VSVG such that high values indicate strong transport to the plasma membrane. HeLa cells were infected and maintained at a non-permissive temperature of 40°C for 3 hours followed by incubation at 20°C for 2 hours to ensure transfer and accumulation of VSVG in the TGN. A subsequent shift to the permissive temperature of 32°C was then used to selectively monitor TGN to plasma membrane transport. In accordance with what has previously been shown, knockdown of FAPP2 using two independent siRNAs led to significant inhibition of VSVG transport from the TGN to the plasma membrane, compared to cells transfected with the siSR control (Figure 5). Different time points after transfer to the permissive temperature were examined and the most evident difference was seen after 45 minutes (data not shown) in accordance with what has been shown previously (Godí et al., 2004).

To test if rescue of FAPP2 induced autophagy affects plasma membrane transfer we knocked down ATG7 in combination with FAPP2 depletion and found that levels of plasma membrane localized VSVG were similar to those seen for the ATG7 single knockdown (Figure 5). This indicates that the transport inhibition is dependent on autophagy induction rather than on FAPP2 depletion. Interestingly, in these initial phases, knockdown of ATG7 also had some inhibitory effects on the amount of VSVG on the cell surface, although this effect was not additive to the inhibitory effect of FAPP2 knockdown.

We included the two siRNAs against FAPP2, siFAPP2a and siFAPP2b, to ensure that the inhibition was specific to FAPP2 removal and not caused by knockdown of other cellular targets. siFAPP2b showed stronger inhibition of transport than siFAPP2a, indicating siFAPP2b knockdown to be slightly
stronger. This indicates that their abilities to block transport correlate with the degrees to which they induce autophagy, and that siFAPP2 mediated inhibition of transport is dependent on the induction of autophagy.

To ensure that the ATG7 rescue of transport in FAPP2 depleted cells was not due to a secondary effect, we conducted similar assays of VSVG transport in SK-BR-3 breast cancer cells. As expected, there was an intracellular accumulation of VSVG-GFP after incubation at non-permissive temperature and a strong export of VSVG-GFP to the plasma membrane upon incubation at permissive temperature (Figure 6A). In accordance with previous studies (Godi et al., 2004; Vieira et al., 2005), knockdown of FAPP2 inhibited the transport of VSVG-GFP to the plasma membrane and resulted in elevated levels of intracellular VSVG-GFP. Importantly, in the SK-BR-3 cells as in the HeLa cells, we found that double knockdown of FAPP2 and ATG7 rescued the inhibitory effect on VSVG-GFP surface transport seen with FAPP2 single knockdown (Figure 6A and B). These findings indicate that while FAPP2 removal results in decreased cargo transfer, FAPP2 is not critical for secretion because removal of both FAPP2 and ATG7 in the same cell normalizes export.

We then employed an alternative approach to minimize potential discrepancies due to differences in experimental conditions and cell lines. In this approach we first triggered autophagy using Rapamycin, an inhibitor of the nutrient sensing mTOR-Raptor complex (Cutler et al., 1999), and looked for changes in the surface expression of the major histocompatibility complex class I chain-related gene A (MICA) fused to GFP that reaches the surface via the trans-Golgi network (Skov et al., 2005). Using flow cytometry, total cellular MICA expression (GFP) was distinguished from cell surface expressed MICA (stained with APC-coupled anti-MICA Ab). As shown in Supplemental Figure 5 A, Rapamycin treatment caused robust inhibition of MICA surface expression. This suggests that increased autophagy can dampen protein secretion via the secretory pathway. To corroborate this, we transfected Jurkat cells with siFAPP2 and/or siATG7 and measured the effect on MICA surface expression. In agreement with earlier reports (Godí et al., 2004; Vieira et al., 2005), and like Rapamycin treatment, FAPP2 depletion down regulated MICA surface expression by ~20% (Supplemental Figure 5 B). Importantly, MICA surface expression was re-established in cell co-transfected with siFAPP2 and siATG7. We speculate that the slight increase in MICA surface expression upon ATG7 depletion is caused by inhibition of an intrinsic level of autophagy in Jurkat cells.
These results indicate that autophagy diverts cell surface proteins from reaching the plasma membrane upon FAPP2 depletion, and that protein export from Golgi to the cell surface is independent of FAPP2.

**FAPP2 localizes to ATG5 positive structures upon autophagic induction**

Given the connection between FAPP2 and autophagy, we examined the subcellular localization of FAPP2 upon autophagy induction. It has previously been shown that FAPP2 is associated with the late Golgi, but we found that it delocalized to discrete, punctuate structures in HeLa cells treated with Rapamycin, (Figure 7 second column). We then assesse d whether these foci might represent structures related to the initiation or completion of autophagosomes.

Since it is well established that ATG5 conjugates with ATG12 and localizes to the early expanding phagophore (Mizushima et al., 2001; Suzuki et al., 2001), we co-transfected HeLa cells with FAPP2-GFP and ATG5-mCherry and examined their subcellular distribution. In untreated cells, FAPP2 localized to the Golgi while ATG5 was primarily cytosolic, and no co-localization was seen (Figure 7 first column). However, upon autophagy induction by Rapamycin, almost all ATG5-mCherry appeared to be associated with vesicles containing FAPP2-GFP (Figure 7 second column). Interestingly, this accumulation of FAPP2-GFP and co-localization with mCherry-ATG5 seems to require the conjugation of ATG5 and ATG12 because expression of a dominant negative mutant forms of ATG5, mCherry-ATG5K130R(Pyo et al., 2005), kept FAPP2-GFP associated with the Golgi with no overlap in localization with ATG5K130R (Figure 7, third and fourth columns).

**DISCUSSION**

Autophagy is a conserved pathway for degradation of bulk proteins and cytoplasmic components first discovered in yeast (Klionsky and Emr, 2000). Many different types of stresses and endogenous processes affect autophagic activity (Delgado et al., 2008; Hofius et al., 2009; Liu et al., 2009; Sumpter and Levine, 2010). Nonetheless, autophagic regulators downstream of these stimuli are still largely unknown. Here we show that autophagy is induced upon FAPP2 depletion. In correlation with previous reports, we also find that transport from the Golgi to the plasma membrane is inhibited upon FAPP2 removal (Godri et al., 2004; Vieira et al., 2005). However, we find that this transport is restored when autophagy is inhibited before FAPP2 depletion. In addition we find that FAPP2 associates with ATG5
positive foci in response to Rapamycin. These findings indicate that the function for FAPP2 in secretion is indirect and that FAPP2 may function as a novel regulator of the ancient autophagy pathway.

While our study corroborates earlier reports, it also raises new questions about the in vivo function(s) of FAPP2. We show that the Golgi to plasma membrane transport defects previously reported by us and others (Godi et al., 2004; Vieira et al., 2005) can be attributed to enhanced autophagy because transport is re-established when the autophagic components ATG7 or Beclin1 are removed concomitantly with FAPP2 depletion (Figure 5 and 6). Thus, FAPP2 function seems to be dispensable for Golgi to plasma membrane transport. Our data show that stronger induction of autophagy leads to stronger inhibition of Golgi to plasma membrane transport (Figure 5). This indicates that inducted autophagy levels correlate with transport blockage. These data are supported by our demonstration of decreased protein export upon autophagy activation by Rapamycin.

It may not be surprising that cells with increased autophagy have decreased export from the Golgi to the plasma-membrane. For example, it is possible that vesicles destined to reach the plasma-membrane become entrapped by expanding autophagosomes. In addition or alternatively, parts of the cellular machinery driving vesicle formation may become more committed to autophagy with a concurrent reduction in the level of export. Other reports have shown that cargo transport from the Golgi to the plasma membrane is attenuated upon starvation accompanied by autophagy induction (Shorer et al., 2005). Thus, autophagy induction may reduce conventional secretion.

Our localization studies show that FAPP2 localizes to ATG5 positive foci upon autophagy induction and that this requires conjugation of ATG5 and ATG12 (Figure 7). This suggests that FAPP2 could regulate autophagy, for example during the initiation of autophagosome formation when ATG5 conjugates with ATG12. Cao and co-workers assigned a new function to FAPP2 and showed that it can form tubules from membrane sheets in vitro (Cao et al., 2009). Other proteins, like the endophilin Bif-1, also have tubulation activities and Bif-1 co-localizes with ATG5 to positively regulate autophagy (Takahashi et al., 2007). Since FAPP2 can drive tubulation from flat membrane sheets (Takahashi et al., 2007), FAPP2 might also be a regulator of autophagosome biogenesis. This is particularly interesting because FAPP2 is Golgi associated and the Golgi plays critical roles in supplying lipids
required for the biogenesis of autophagosomal like vesicles (van der Vaart et al., 2010). Nonetheless, FAPP2 is not essential for autophagy induction since autophagy is activated in its absence. Instead, the GLTP homolog of the FAPP2 sphingolipid binding domain could drive similar processes when FAPP2 is absent. In this context it is noteworthy that double depletion of FAPP2 and GLTP is lethal (Halter et al., 2007).

While it is formally possible that FAPP2 negatively regulates autophagy, an alternative explanation for the induction of autophagy upon FAPP2 depletion derives from our discoveries in plants. We demonstrated that loss of Arabidopsis ACD11 triggers a plant immune receptor homologous to mammalian cytosolic NOD-like receptors (NLRs) (Palma et al., 2010). In plants as in animals, successful pathogens deliver effectors into cells which modify or remove host proteins to suppress immune responses. These modifications are perceived by plant NLRs that trigger immune responses (Dangl et al., 2013). Since the absence of ACD11 triggers immunity via an NLR in Arabidopsis, ACD11 (or a complex or pathway containing ACD11) may be targeted by pathogen effector molecules. Interestingly, the FAPP2 interactor ARF1 is targeted by the Shigella flexneri cysteine effector IpaJ. In addition, ARF1 has been linked to autophagy as uncontrolled Arf1 activation strongly inhibits mTORC1 activity (Li et al., 2010) and ARF1 is required for lipidation of ATG8/LC3 in yeast (van der Vaart et al., 2010). Since both mammalian NODs and Arabidopsis NLRs induce autophagy (Hofius et al., 2009; Travassos et al., 2010), it is possible that a FAPP2 ARF1 complex represents an effector target under surveillance by innate immunity receptors.

**MATERIALS AND METHODS**

**HeLa cultivation, transfection and treatment**

HeLa cells were cultured in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100μg/ml streptomycinin 5% CO₂ and humidity saturation at 37°C. For transfections cells were plated on coverslips in 6-well plates. When cells were 70-80% confluent, transfections were carried out using Lipofectamine™ 2000 (Invitrogen) according to
manufacturer’s recommendations. 24-72 hours after transfection cells were fixed in 2-4% paraformaldehyde (PFA) in PBS.

For starvation 24 hours post transfection, cells were first washed with PBS three times and then incubated in HBSS (Invitrogen) for 4 hours before fixation. Cells treated with Bafilomycin (Sigma) were, 24 hours post transfection, washed three times with PBS and incubated in DMEM supplemented with 100nM Bafilomycin for 4 hours.

**GFP-LC3 confocal microscopy and quantification in HeLa cells**

Images of HeLa cells co-transfected with siRNA directed against FAPP2 and either WT FAPP2 or the siRNA-resistant version were obtained using a Leica SP5-X inverted point-scanning confocal microscope. A 488nm Argon Ion laser was used for detection of GFP. Transfected cells subjected to starvation or treatment with Bafilomycin were also imaged on the Leica SP5-X confocal microscope. Images of HeLa cells co-transfected with siRNA directed against FAPP2 and ATG7 or Beclin1 were obtained using a Zeiss LSM 700 confocal microscope. Image processing was done using the Zen12 light software (Zeiss). For quantification of autophagosomes, GFP-LC3 positive foci were counted from triplicates in more than 20 cells. Quantification was performed with ImageJ generating a cut-off for the basal dispersed LC3 fluorescence, allowing for the quantification of fluorescence in concentrated areas (GFP-LC3 -associated autophagosomes). Quantification of GFP-LC3 puncta per cell was performed by counting in a blinded experiment.

**Jurkat cultivation, transfection and treatment**

Jurkat cells were grown in RPMI 1640 supplemented with 10% FBS, 2mM glutamine, 2mM Penicillin and streptomycin. Cells were incubated in 5% of CO2 and humidity saturation at 37°C. Jurkat Tag cells were transiently transfected with the Nucleofactor™ kit (Amaxa Biosystems) according to manufacturer’s protocol, using #G10. Jurkat cells used for MICA transport assays were treated with varying concentrations of Rapamycin for 2 hours.

**GFP-LC3 confocal microscopy and quantification in Jurkat cells**
Data acquisition and cytometric analysis were performed on a BD FACSCalibur using Cellquest software. Cells used for confocal analysis were fixed in 2-4% paraformaldehyde (PFA) in PBS 24 hours after transfection. Quantification was performed as for HeLa cells.

**Constructs used**

For generation of a siRNA-resistant versions of FAPP2 3 × Flag-FAPP2-519 (D'Angelo et al., 2007) were mutagenized to produce mismatches in the duplex region without inducing any amino-acid change in the protein sequence. The DNA sequence in the duplex region after mutations was (mutated bases in bold): 5'-CATTCCTCGCTTCGTGTTA-3'.

GFP-LC3 was kindly provided by Tamotsu Yoshimori (Kabeya et al., 2000).

cDNA of FAPP2 was amplified by PCR with primers FAPP2.F (5'-CTGCTAGCATGGAGGGGTGCTGTACAGTGGACCAACT-3') and FAPP2.R (5'-GTGGATCCGCTAAAGCGTCCAGTATGGCCATCTGTTTC-3'). For generation of FAPP2-GFP the amplified sequence were cloned into the *Nhe*I and *Bam*HI sites in pEGFP-N1. For co-localization studies pmCherry-ATG5 (addgene #13095) and pmCherry-ATG5-K130R (addgene #13096) (Hamacher-Brady et al., 2007) were used in combination with the generated FAPP2-GFP.

**siRNAs**

Target sequence for siRNA directed agains t FAPP2a: 5’-GAATTGATGTGGGAACTTT -3’, FAPP2b: 5’-GCATTCTTGACATCATG-3’, FAPP2v: 5’-GACTCTACTGTGACCTCCT-3’ (Godi et al., 2004), ATG7: 5’-GGAGTCACAGCTCTTCCTT-3’ (Yu et al., 2004) and Beclin1: 5’-CAGTTTGGCACAAATCAATA-3’ (Yu et al., 2004). All siRNAs were synthesized by Qiagen. As control the non-silencing oligonucleotide siSR (#1022076, Qiagen) was used. This control siRNA consists of a pool of scrambled siRNA with at least four mismatches for all sequences in the human genome.

**Immunoblot**

Protein was extracted in RIPA buffer (50mM Tris pH 7.5 or 8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, Protease Inhibitors), after cells were washed in cold 1X PBS at least once.
SDS-PAGE gels were prepared with 10, 12 or 6-14% gradient cross linking. Proteins were transferred with electro-blotting onto PVDF membrane. Membranes were rinsed in TBS and blocked for 1 h in 5% (w/v) non-fat milk in TBS-Tween (0.1% (v/v)). Membranes were incubated with primary antibodies for 1 h to overnight. Membranes were washed 3 × 10 min in TBS-T (0.1%) before 1-h incubation with HRP-conjugated secondary antibodies. Chemiluminescent substrate (ECL Plus, Pierce) was applied before exposure to film (AGFA CP-BU) or camera recording. For probing immunoblots with multiple antibodies, stripping was carried out using Restore Western Blot Stripping Buffer (Pierce) for 15–30 min, followed by three washes with TBS-Tween.

**Antibodies**

Polyclonal anti-GM130 FAPP2 was raised in rabbits as a glutathione S-transferase fusion protein and then affinity purified. Rabbit anti-GFP antibody was from OriGene Technologies Inc., and antibody against ERK1/2 was from Santa Cruz Biotechnology. Antibodies against β-actin, FLAG and P5D4 anti-VSVG(for total VSVG), were from Sigma, and anti-LC3-B antibody was from Novus Biochemicals. Antibody to the VSVG luminal domain was from J. Gruenberg, U. of Geneva, Switzerland. Alexa-555-conjugated Herceptin antibody from Genentech was used to stain the membrane in SK-BR-3 experiments.

**RT-qPCR**

Total RNA was extracted from Jurkat cells 48hours post siRNA treatment using RNeasy protect kit (Qiagen). RNA integrity was confirmed by agarose gel electrophoresis. Samples were subjected to quantitative PCR using Brilliant SYBR Green QPCR Master Mix kit (Stratagene). FAPP2 and B-actin were quantified separately in triplicates. Primer sequences were FAPP2.F2 and R2 (5’-GGCAATGGAGTGGACACTTG-3’, 5’-TTCGTGACACTATCTCTCTG-3’).

**Transmission electron microscopy (TEM)**

Jurkat cells were fixed in a mixture of 2% PFA and 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7, overnight at 5°C. After a short rinse in buffer, post-fixation was carried out for 2 hours at room temperature in 1% OsO₄. After rinsing in buffer and distilled water, dehydration was done in a graded series of acetone before mixing and infiltration with Spurr’s embedding mixture (Bozzola, 1999).
cell suspension was centrifuged at 200G for 5 min at every change in solution from the fixation to the infiltration process. The pellet was then sedimented into gelatin capsules with Spurr’s resin before polymerization at 70°C. Ultrathin (50-100 nm) sections were cut with a diamond knife on a Reichert-Jung Super Nova ultramicrotome, collected on slot-grids with formvar film and stretched by chloroform vapor. The sections were contrasted with uranyl acetate and lead citrate (Bozzola, 1999) and examined in a Jeol 1010CX transmission electron microscope, equipped with a Gatan digital camera.

**VSVG Golgi-PM transport (HeLa cells)**

Analysis of VSVG protein transport was performed in HeLa cells infected with VSVG made by Elena Polishchuk as previously described (Polishchuk et al., 2000). Cells, plated on coverslips, were infected with VSVG at 32 °C for 45 min, incubated at 40 °C for 3 h, and then incubated at 20°C for 2 hours (to permit accumulation in TGN) before being shifted to 32 °C for different times. At the end of the incubations, the cells were fixed in 4% PFA and processed for immunofluorescence and quantification of transport.

Cell profiler (Carpenter et al., 2006) was used to identify individual cells from DAPI stain, and to calculate the integrated value from the green channel (internal) and the red channel (external) for each cell. Each point represents the ratio of surface/whole cell fluorescence of a single cell. Images were visually inspected to ensure the precision of the program. Samples for confocal imaging were examined under a Zeiss LSM 510 confocal laser scanning microscope.

**VSVG-GFP ER-PM transport (SK-BR-3)**

SK-BR-3 cells were grown at 37°C, 5% CO2 and 98% humidity in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS), 2mM glutamine, 200U/ml penicillin and 50ng/ml streptomycin. 24 hours after plating, SK-BR-3 cells were co-transfected with VSVG-GFP and various siRNA using TranspassR1 (NEB). siRNA amounts were equal in all transfections by addition of siSR.

96 hours after plating, transfected SK-BR-3 cells were incubated at 39.5°C for 4 hours, causing VSVG-GFP to accumulate in the ER, then at 32°C for 4 hours to allow VSVG-GFP to be transported to the plasma membrane. Cells were fixed in 2% PFA and stained with Alexa-555-conjugated Herceptin
antibody. Microscopy of SK-BR-3 cells used a Zeiss LSM510 Meta microscope, and samples were randomized and their identity remained unknown during microscopy, image processing and image analysis. Images were sectioned into smaller fractions containing only a single cell using Adobe Photoshop, whereas all further handling was done in ImageJ using the MBF Biophotonics Plug-in compilation. The relative amount of VSVG-GFP in the plasma membrane was then calculated by dividing the integrated amount of VSVG-GFP fluorescence within the plasma membrane mask with the integrated VSVG-GFP fluorescence within the cell mask. The method for quantification of VSVG-GFP in the plasma membrane is described in detail in Supplemental Figure 4.

**MICA transport**

Jurkat cells transfected with MICA-GFP (Mellergaard et al., 2014) were either treated with Rapamycin for 2 hours at different concentrations or co-transfected with indicated siRNAs. Cells were analyzed 24 hours after transfection. Flow cytometry was used to measure the surface expression of MICA (APC coupled anti-MICA Ab) and total MICA (GFP) expression. Data acquisition and cytometric analysis were performed on a BD FACSCalibur using Cellquest software. Since the different staining’s cannot be directly compared, relative difference between surface and total MICA expression were calculated.
References


Chea, E., Le Bourhis, L., et al. (2010). Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma
vander Vaart, A., Griffith, J., and Reggiori, F. (2010). Exit from the Golgi is required for the expansion of the
Figure 1 Accumulation of GFP-LC3 positive foci upon FAPP2 depletion

(A) Removal of FAPP2 in HeLa cells leads to accumulation of GFP-LC3 foci. HeLa cells 24 hours post transfection with either siRNA against FAPP2 or control siSR in combination with GFP-LC3. Cells were fixed in 4% PFA, and representative confocal images are shown. The scale bar corresponds to 10µm.

(B) siFAPP2 transfected cell accumulate significantly more GFP-LC3 foci than control cells. Quantification of GFP-LC3 puncta. Cells were treated as in A. Data (mean±SD) are representative of at least three independent experiments. Asterisks indicate statistical significance, P<0.05 by one-way ANOVA followed by Tukey’s HSD test.

(C) Enhanced levels of LC3-GFP and cleaved GFP are detected upon FAPP2 depletion. Immunoblot analysis of cells co-transfected with either siSR or siFAPP2 in combination with GFP-LC3. Blots were probed with anti-GFP antibodies. Probing with anti β-actin probing was used for loading control.
Figure 2 Accumulation of LC3 foci after FAPP2 knock-down is specific

(A) No reduction in FAPP2-FLAG was detected in cells co-transfected with siFAPP2b and the mutated form of FAPP2. HeLa cells were transfected with FAPP2-WT or a mutated version of FAPP2 in combination with scrambled siSR or siRNA directed against FAPP2 as indicated. Total lysates were subjected to immunoblot analysis with anti-FLAG antibodies. Anti-ERK1/2 was included as a loading control.

(B and C) FAPP2 knock-down induced GFP-LC3 accumulation rescued in cells complemented with a siFAPP2b resistant version of FAPP2. (B) Representative confocal images of cells treated as in (A). Cells were co-transfected with GFP-LC3 and fixed in 4% PFA 24 hours post transfection.

(C) Number of GFP-LC3 puncta per cell was quantified in cells from (B). Data (mean±SD) are representative of at least three independent experiments. Asterisks indicate statistical significance, P<0.05 by one-way ANOVA followed by Tukey’s HSD test.
Figure 3 Knock-down of FAPP2 induces ATG7 dependent autophagy

(A and B) Accumulation of GFP-LC3 foci upon FAPP2 depletion is rescued by removal of the autophagy components ATG7 or Bec1.

(A) HeLa cells were transfected with scrambled siSR or siFAPP2 in combination with siSR, siATG7 or siBec1. All cells were also co-transfected with GFP-LC3. Cells were fixed in 4% PFA 24 hours post transfection followed by imaging, representative images are shown. The scale bar corresponds to 10µm.

(B) Quantification of GFP-LC3 labelled autophagosomes per cell. Number of GFP-LC3 puncta per cell was counted in cells from (A). Data (mean±SD) are representative of at least three independent experiments. Asterisks indicate statistical significance, P<0.05 by one-way ANOVA.

(C) LC3 conversion upon FAPP2 knock-down is reduced in siFAPP2/siATG7 co-transfected cells. Immunoblot analysis of cells transfected with siSR, siFAPP2 or siATG7. Blots were probed with anti-FAPP2 and LC3-II antibodies. Anti β-actin probing were included for loading control.
**Figure 4** Knock-down of FAPP2 results in increased autophagic flux

(A and B) Starvation following FAPP2 knock-down does not affect foci accumulation while treatment with BAF increases foci accumulation.

(A) Representative confocal images of HeLa cells transfected with scrambled siSR, siFAPP2v or siFAPP2b. 24 hours after transfection cells were either subjected to starvation in HBSS or treated with 100mM Bafilomycin for 4 hours. Cells were then fixed in 4% PFA and analyzed. Control cells cultured in DMEM are included.

(B) Quantification of GFP-LC3 positive foci in cells from (A). A significantly higher number of GFP-LC3 foci were seen in cells transfected with siRNA directed against FAPP2 and treated with Bafilomycin. Data (mean±SD) are representative of at least three independent experiments. Asterisks indicate statistical significance, P<0.05 by one-way ANOVA.
Figure 5 FAPP2 is not critical for TGN to plasma membrane transport in HeLa cells.
Reduced transport from Golgi to the plasma membrane due to FAPP2 knock-down is rescued upon autophagy inhibition.
Control, siFAPP2 and siFAPP2/siATG7 doubly treated HeLa cells were infected with ts045-VSVG, incubated at 40°C for 3 hours, then at 20°C for 2 hours to allow VSVG accumulation in the Golgi, then shifted to 32°C to allow VSVG transport to the plasma membrane, and finally processed for transport analysis. Transport was calculated as the ratio of the integrated value of staining for plasma membrane VSVG relative to whole cell VSVG. High values therefore indicate strong transport to the plasma membrane. All comparisons were tested and only cells transfected with siRNA directed against FAPP2 had a significantly lower surface/whole cell ratio. P<0.01 by ANOVA followed by Tukey’s HSD test.
**Figure 6** FAPP2 knock-down inhibits export to the plasma membrane due to increased autophagy in SK-BR-3 cells.

(A and B) Plasma membrane transport of VSVG-GFP is inhibited in siFAPP2 treated SK-BR-3 cells, but restored in siFAPP2/siATG7 doubly transfected cells.

(A) Confocal images of SK-BR-3 cells. Control, siFAPP2 and siFAPP2/siATG7 doubly treated SK-BR-3 cells were infected with ts045-VSVG-GFP, maintained at 40°C for 4 hours, shifted to 32°C to allow VSVG-GFP transport to the plasma membrane, and then fixed and processed for immunofluorescence microscopy with a Alexa-555-conjugated Herceptin plasma membrane marker. Scale bars correspond to 20nm.

(B) Automated quantification of VSVG-GFP transport to the plasma membrane in cells treated as in A. See Supplemental figure 4 for details on the quantification. Bars = 95% CI, asterisk = P<0.005 in Mann-Whitney U-test (n>70).
Figure 7 FAPP2 co-localizes with ATG5 positive foci upon autophagy induction. FAPP2 co-localizes with ATG5 in response to rapamycin, but co-localization is not detected with dominant negative mATG5K130R. HeLa cells, co-transfected with FAPP2-GFP and mCherry-ATG5 or the dominant negative mutant mATG5K130R for 48 hours, were incubated for two hours with or without 10µM Rapamycin, before being fixed in 2% PFA. Representative confocal microscope images are shown. Merged pictures show overlay of GFP and mCherry channels, and the yellow color indicates co-localization. The bottom panels are enlarged from the boxed areas in the merged pictures. The scale bar corresponds to 25µm.
**Supplemental Figure 1** Accumulation of autophagosomes upon FAPP2 depletion in Jurkat cells

(A) FAPP2 knock-down results in accumulation of GFP-LC3 positive foci. Quantification of Jurkat cells 24 hours post transfection with either siRNA against FAPP2 or control siSR in combination with GFP-LC3. Cells were fixed in 4% PFA 24 hours post transfection and analyzed by confocal microscopy. Data (mean±SD) are representative of at least three independent experiments.

(B) FAPP2 transcription levels are markedly reduced upon siFAPP2 treatment. Jurkat cells treated with either control siSR or siRNA against FAPP2 were used for mRNA extraction 24 hours post transfection. FAPP2 mRNA levels determined by qPCR using the housekeeping gene Ribosomal Protein Large P0 (RPL0) as an internal standard. Standard deviation of the mean is indicated by error bars. The experiment was performed with triplicate biological samples, each with technical triplicates.

(C) Representative TEM images of Jurkat cells 24 hours post transfection with siSR or siFAPP2 as indicated. The bottom panels are enlarged from the boxed areas. The arrow indicates early and the arrowhead late autophagosomes. Scale bars corresponds to 1µm (top panels) and 0.51µm (bottom panels).
**Supplemental Figure 2** GFP-LC3 accumulation by FAPP2 KD is ATG7 and Bec1 dependent in Jurkat cells

(A and B) Accumulation of GFP-LC3 foci upon FAPP2 depletion is also rescued by removal of the autophagy components ATG7 and Bec1 in Jurkat cells.

(A) Jurkat cells co-transfected with different siRNAs as indicated in combination with GFP-LC3. Cells were analyzed 24 post transfection by confocal microscopy, representative images are shown.

(B) Quantification of GFP-LC3 labelled foci per cell. Number of GFP-LC3 foci per cell was counted in cells from (A). Data (mean±SD) are representative of at least three independent experiments.
Supplemental Figure 3 Depletion of FAPP2 induces autophagic flux

Treatment with Bafilomycin of FAPP2 depleted HeLa cells results in slightly increased accumulation of LC3-II indicating that FAPP2 depletion leads to increased autophagic flux. Immunoblot analysis of HeLa cells transfected with either siSR or siFAPP2 and treated as indicated. Blots were propped with anti-FAPP2 antibody to ensure proper FAPP2 KD and with anti-LC3-II as a measure for LC3 conversion. Anti β-actin probing were included as control for equal loading.
**Supplemental Figure 4** Example of the quantification of VSVG-GFP at the plasma membrane.

(A) Original image represents a confocal image of a SK-BR-3 breast cancer cell transfected with the temperature sensitive VSVG (tsO45) fused to GFP (VSVG-GFP, green) and immunostained with the plasma membrane marker Alexa-555 conjugated Herceptin (red). Yellow color indicates co-localization. Monochromatic images in top row show the VSVG-GFP and plasma membrane signal individually. A cell mask as indicated was generated in an automated fashion, the VSVG-GFP fluorescence in the region was integrated and used in the quantification to estimate the total VSVG-GFP in the cell (first image, bottom row). The mask was generated in ImageJ from average signal of VSVG-GFP and plasma membrane marker using Median filtering \((r=2)\) and the Mixture Modeling Threshold plugin. A plasma membrane mask was generated; the VSVG-GFP fluorescence in the region was integrated and used to estimate the amount of VSVG-GFP at the plasma membrane (mid image, bottom row). The last image in the bottom row shows the VSVG-GFP channel with an overlay of the borders of the cell mask (blue) and the plasma membrane mask (red). The relative amount of VSVG-GFP at the plasma membrane was then calculated by dividing the integrated amount of VSVG-GFP fluorescence within the plasma membrane mask with the integrated VSVG-GFP fluorescence within the cell mask.

(B) Graph showing the percentiles of the quantification shown in Figure 6.
Supplemental Figure 5 Autophagic activity leads to decreased protein export

(A) Induction of autophagy by Rapamycin treatment inhibits export of MICA. Jurkat cells transfected with MICA-GFP were treated with Rapamycin at the indicated concentrations for two hours. Flow cytometry was used to measure surface expression of MICA (APC coupled anti-MICA Ab) and total MICA (GFP) expression. As different staining’s cannot be directly compared, results show the relative difference between surface and total MICA expression.

(B) Decreased protein transport caused by FAPP2 depletion is rescued by inhibition of autophagy. Concomitantly with MICA-GFP Jurkat cells were transfected with the siRNAs as indicated. Cells were analyzed 24 hours after transfection as described above.