



# Constitutive Negative Regulation of R Proteins in *Arabidopsis* also via Autophagy Related Pathway?

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Even though resistance (R) genes are among the most studied components of the plant immunity, there remain still a lot of aspects to be explained about the regulation of their function. Many gain-of-function mutants of R genes and loss-of-function of their regulators often demonstrate up-regulated defense responses in combination with dwarf stature and/or spontaneous leaf lesions formation. For most of these mutants, phenotypes are a consequence of an ectopic activation of R genes. Based on the compilation and comparison of published results in this field, we have concluded that the constitutively activated defense phenotypes recurrently arise by disruption of tight, constitutive and multilevel negative control of some of R proteins that might involve also their targeting to the autophagy pathway. This mode of R protein regulation is supported also by protein–protein interactions listed in available databases, as well as *in silico* search for autophagy machinery interacting motifs. The suggested model could resolve some explanatory discrepancies found in the studies of the immunity responses of autophagy mutants.

**Keywords:** resistance, autophagy, R, Avr, ETI, dwarf, lesions, exocyst

## INTRODUCTION

There are several approaches how to study and classify the plant immunity related events, and the most widespread is division of the plant immunity into two modes – a pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI), which is triggered usually by recognition of structural components of pathogen on the surface of the host cell, and effector triggered immunity (ETI; Jones and Dangl, 2006). These two defense modes employ basically the same means, but PTI is more general and mild, while ETI is much stronger and more efficient. ETI is triggered by the direct or indirect interaction between a specific disease resistance (R) protein and a corresponding avirulence (Avr) protein of pathogen and is accompanied by a number of changes within the plant – production of reactive oxygen species (ROS) by an oxidative burst, accumulation of the salicylic acid (SA), and the transcriptional activation of genes involved in defense response, that lead to a possible final stage – localized programmed cell death called the hypersensitive response (HR; Pontier et al., 1998; review in McDowell and Woffenden, 2003; Vlot et al., 2008).

Disease resistance (R) genes are central components of the plant immune response. All R proteins contain at least some of basic motifs – either Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) structure on the N terminal part, nucleotide-binding site (NBS), leucine-rich repeat

(LRR), protein kinase and transmembrane domains (review by Martin, 1999; Liu et al., 2007). There are 145 putative genes encoding a product with a TIR domain and 51 with CC domain predicted in the *Arabidopsis thaliana* Col-0 genome (Meyers et al., 2003; Jacob et al., 2013). Majority encode proteins with TIR, NBS, and LRR domains, making the TNL group; some genes encode proteins with TIR and NBS domains but no LRR domain (TN genes) and some encode proteins with a TIR domain only (TX genes; Meyers et al., 2003; Nandety et al., 2013). Besides CC-NBS-LRR containing proteins which make CNL group, there are also four proteins that have NBS motifs similar to CNLs, but lack a CC motif (Meyers et al., 2003).

There are several important molecules involved in signaling downstream the successful R-Avr recognition – ENHANCED DISEASE SENSITIVITY 1 (EDS1), PHYTOALEXIN DEFICIENT 4 (PAD4), NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1) and SENESCENCE ASSOCIATED GENE 101 (SAG101), which are essential for the accomplishment of HR and for the accumulation of the SA. EDS1, PAD4 and SAG101 are involved in transferring signals mainly from TNL proteins, while CNL pathway mostly relies on signaling through NDR1 (Century et al., 1997; Feys et al., 2001; He and Gan, 2002; Wagner et al., 2013).

In *Arabidopsis* mutants in genes coding for R and R-associated proteins, along with defense related deviations, two other most frequent phenotypes are a dwarf stature and a spontaneous HR lesion formation; many times present even simultaneously (Table 1). Rarely, a lethal phenotype occurs as well, even though no developmental function for these genes has been found so far. We could notice that for most of the R genes mutants, described phenotypes are a consequence of their activation, in some cases even a gain of function mutations (GOF). Based on the comparison of different studies of plant immunity, our hypothesis aims to suggest a model in which the hyper immune phenotypes arise as a result of disruption of tight, multistep and constitutive negative control of R proteins that possibly involves also their inactivation by the autophagy pathway.

## OF DWARFS AND LESIONS

It was shown that mutants with over activated R protein dependent defense response develop mostly two phenotypes – dwarfism and/or necrotic leaf lesions (reviewed e.g., in Lorrain et al., 2003 and Janda and Ruelland, 2014). For instance, in plants overexpressing a CNL gene *ACTIVATED DISEASE RESISTANCE 1* (*ADR1*), a constitutive defense response and a dwarf phenotype were found (Grant et al., 2003). A TNL protein *SUPPRESSOR OF NPR1 CONSTITUTIVE 1* (*SNC1*) was found to be overactive in the *bonzai1-1* (*bon1-1*) mutant which also shows a constitutive defense response and reduced plant size (Yang and Hua, 2004). Along with *bon1*, several other autoimmune dwarf mutations were found to be suppressed by mutation of *SNC1* locus; namely in *BON1-ASSOCIATED PROTEIN* (*bap1*), *BAK1-INTERACTING RECEPTOR-LIKE KINASE 1* (*bir1*), *SUPPRESSOR OF RPS4-RLD 1* (*srfr1*), *CONSTITUTIVE EXPRESSER OF PATHOGENESIS-RELATED GENE* (*cpr1*) and

*MITOGEN-ACTIVATED PROTEIN KINASE 1* (*mpk1*; review in Gou and Hua, 2012). Plants overexpressing a TIR-X gene *At2g32140* show also dwarf phenotype and activated expression of defense-related genes (Kato et al., 2014). This phenotype was dependent on EDS1, PAD4, and partially dependent on *SALICYLIC ACID INDUCTION DEFICIENT 2* (*SID2*).

HR-like spontaneous leaf necrotic lesions were found to be even more frequently associated with the mutations in R genes and constitutively activated immunity. For instance, a GOF mutant in TNL *RPP4* locus called *chilling sensitive 2* (*chs2*) shows lesions in the low temperature conditions (Huang et al., 2010). GOF *Arabidopsis* mutant in the other *CHS* gene, *chs3-1*, which encodes an unconventional disease resistance (R) protein belonging to the TIR-NB-LRR class with a zinc-binding LIM domain (Lin-11, Isl-1 and Mec-3 domains) at the carboxyl terminus, shows arrested growth, chlorosis and constitutively activated defence responses at 16°C (Yang et al., 2010). A mutant in TNL gene *ssi4* develops chlorotic lesions which can be suppressed by high humidity (Shirano et al., 2002; Zhou et al., 2004). In addition, there are several examples of mutants with spontaneous lesions induction which are suppressed by mutations in loci encoding R proteins of CNL type – *ACTIVATED DISEASE RESISTANCE 1* – *adr1*, *adr1-11* and *adr1-12* suppress *LESION SIMULATING DISEASE 1* (*lsd1*) by down regulating SA signaling (Bonardi et al., 2011; Roberts et al., 2013). Likewise, when a putative TNL encoded by *LAZARUS 5* (*LAZ5*) gene is mutated, *accelerated cell death 11* (*acd11*) lesion phenotype can be suppressed (Palma et al., 2010). It was also shown, that in the absence of the copine-like proteins *BON1* and *BON3* function, several R-like genes of the TNL/TN type were found to trigger lesion cell death (LCD; Li et al., 2009). Mutation in *SUPPRESSOR OF MKK1 MKK2 2* (*summ2*) which encodes putative NB-LRR, suppresses lesions formation and dwarfism of mutants of MAP kinase pathway *mkk1/mkk2* and *mpk4* (Kong et al., 2012).

There are genes coding for other defense related components that when mutated trigger the same constitutive immunity activation and dwarf or/and lesion mimic phenotypes – e.g., *CONSTITUTIVE EXPRESSER OF PATHOGENESIS-RELATED GENE 1* (*CPR1*), *SUPPRESSOR OF SALICYLIC ACID INSENSITIVITY OF NPR1-5 2* (*SSI2*), *DEFENSE NO DEATH 1* (*DND1*), *TYPE III PHOSPHATIDYLINOSITOL-4-KINASES  $\beta 1\beta 2$*  (*PI4KIII $\beta 1\beta 2$* ) (Bowling et al., 1994; Yu et al., 1998; Zhang et al., 2003; Sekine et al., 2004; Gou et al., 2012; Sasek et al., 2014). As a regular aspect of these mutants' phenotype deviations, hyper accumulation of SA was observed.

## LETHALITY OF THE HUB

Overactive immunity can disturb plant growth and fitness, and in an extreme case, this can be deleterious. Unexpectedly, an embryo lethal phenotype was found for LOF mutation of a defense related gene *RPM1-INTERACTING PROTEIN 4* (*RIN4*). Being evolutionarily conserved protein in plants, *RIN4* is targeted to the plasma membrane by C-terminal acylation, and is required for the activation of a CNL *RESISTANCE TO*

**TABLE 1 | List of *Arabidopsis* mutants related to R proteins hyper activity causing dwarf and lesion mimic phenotypes.**

Gene	Name	Function category	Related mutant phenotypes	Reference
<i>acd11</i>	<i>accelerated cell death 11</i>	Sphingosine transfer protein	Lesions	Brodersen et al., 2002
<i>adr1</i>	<i>activated disease resistance 1</i>	CNL	Lesions suppression, dwarf oe	Grant et al., 2003
<i>adr1-11</i>	<i>activated disease resistance 1-like 1</i>	CNL	Lesions suppression, dwarf oe	Collier et al., 2011
<i>adr1-12</i>	<i>activated disease resistance 1-like 2</i>	CNL	Lesions suppression	Bonardi et al., 2011
<i>atg5</i>	<i>autophagy related gene 5</i>	Autophagy, ubiquitin ligase	Early senescence	Thompson et al., 2005
<i>atg6</i>	<i>autophagy related gene 6/Beclin1</i>	Autophagy activation	Pollen-lethality	Fujiki et al., 2007
<i>atg7</i>	<i>autophagy related gene 7</i>	Autophagy, ubiquitin activating enzyme	Defense-related	Doelling et al., 2002
<i>atg8</i>	<i>autophagy related gene 8</i>	Ubiquitin-like protein, cargo recruitment	/	Ketelaar et al., 2004
<i>bak1</i>	<i>brassinosteroid-insensitive associated 1</i>	Receptor-like protein kinase	Semidwarf	Li et al., 2002
<i>bap1</i>	<i>bon1-associated protein</i>	Calcium-dependent phospholipid-binding	/	Hua et al., 2001
<i>bir1</i>	<i>bak1-interacting receptor-like kinase 1</i>	Receptor-like protein kinase	Dwarf	Gao et al., 2009
<i>bon1</i>	<i>bonzai1</i>	Copine-like, membrane trafficking	Dwarf	Hua et al., 2001
<i>bon2</i>	<i>bonzai2</i>	Copine-like, membrane trafficking	Dwarf	Yang et al., 2006
<i>bon3</i>	<i>bonzai3</i>	Copine-like, membrane trafficking	Dwarf	Yang et al., 2006
<i>chs2</i>	<i>chilling-sensitive 2</i>	TNL	Lesions	Huang et al., 2010
<i>chs3</i>	<i>chilling-sensitive 3</i>	TNL	Lesions	Yang et al., 2010
<i>cpr1</i>	<i>constitutive expresser of pathogenesis-related gene</i>	F-box protein	Dwarf	Gou et al., 2012
<i>dnd1</i>	<i>defense no death 1</i>	Cyclic nucleotide-gated ion channel	Dwarf	Yu et al., 1998
<i>eds1</i>	<i>enhanced disease sensitivity 1</i>	R related signaling	Lesions suppression	Rogers and Ausubel, 1997
<i>exo70A1</i>	<i>exo70A1</i>	Membrane trafficking	Dwarf	Synek et al., 2006
<i>exo70B1</i>	<i>exo70B1</i>	Membrane trafficking	Lesions	Kulich et al., 2013
<i>fls2</i>	<i>flagellin-sensitive 2</i>	Receptor-like protein kinase	Defense related	Gomez-Gomez and Boller, 2000
<i>laz4</i>	<i>lazarus 4</i>	Membrane trafficking	Lesion suppression	Munch et al., 2015
<i>laz5</i>	<i>lazarus 5</i>	R protein	Lesions suppression	Palma et al., 2010
<i>lsd1</i>	<i>lesion simulating disease 1</i>	Cell death related	Lesions	Kliebenstein et al., 1999
<i>mkk1/mkk2</i>	<i>mitogen-activated protein kinase kinase kinase 1/2</i>	Signaling	Dwarf, lesions	Qiu et al., 2008
<i>mpk1</i>	<i>mitogen-activated protein kinase 1</i>	Signaling	Dwarf	Bartels et al., 2009
<i>mpk4</i>	<i>mitogen-activated protein kinase 4</i>	Signaling	Dwarf, lesions	Petersen et al., 2000
<i>ndr1</i>	<i>non-race specific disease resistance 1</i>	R related signaling	Lesions suppression	Century et al., 1995
<i>pad4</i>	<i>phytoalexin deficient 4</i>	R related signaling	Lesions suppression	Jirage et al., 1999
<i>rar1</i>	<i>required for mlo12 resistance 1</i>	R related signaling	Lesions suppression	Azevedo et al., 2002
<i>rin4</i>	<i>rpm1-interacting protein 4</i>	Immunity related	Embryo lethal	Mackey et al., 2002
<i>rpm1</i>	<i>resistance to p. syringae pv maculicola 1</i>	R protein	Defense related	Debener et al., 1991
<i>rps2</i>	<i>resistant to p. syringae 2</i>	R protein	Defense related	Yu et al., 1993
<i>sag101</i>	<i>senescence associated gene 101</i>	R related signaling	/	Feys et al., 2005
<i>sgt1b</i>	<i>suppressor of g-two allele of skp1</i>	R related signaling	Lesions suppression	Azevedo et al., 2002
<i>sid2</i>	<i>salicylic acid insensitive 2</i>	SA synthesis	Defense related	Nawrath and Metraux, 1999
<i>slh1</i>	<i>sensitive to low humidity 1</i>	R protein	Lesions	Noutoshi et al., 2005
<i>snc1</i>	<i>suppressor of npr1 constitutive 1</i>	R protein	Dwarfism suppression	Li et al., 2010
<i>srfr1</i>	<i>suppressor of rps4-rld 1</i>	Tetratricopeptide repeat domain containing	Dwarf	Kim et al., 2010
<i>ssi2</i>	<i>suppressor of SA insensitivity of npr1-5 2</i>	Stearyl-ACP desaturase	Dwarf, lesions	Sekine et al., 2004

(Continued)

TABLE 1 | Continued

Gene	Name	Function category	Related mutant phenotypes	Reference
<i>ssi4</i>	Suppressor of SA insensitivity of <i>npr1-5 4</i>	R protein	Dwarf	Shirano et al., 2002
<i>summ2</i>	Suppressor of <i>mkk1 mkk2 2</i>	R protein	Dwarfism and lesions suppression	Zhang et al., 2012
<i>syp121/syp122</i>	Syntaxin 121/syntaxin 122	Membrane trafficking	Dwarf, lesions	Zhang et al., 2008
<i>syp23</i>	Syntaxin 23	Membrane trafficking	Semi-dwarf	Ohtomo et al., 2005
<i>syp31</i>	Syntaxin 31	Membrane trafficking	/	Chatre et al., 2009
<i>TN2</i>	TIR-NBS 2	R protein	Lesions suppression	Zhao et al., 2015
<i>TX At2g32140</i>	/	R protein	Dwarf	Kato et al., 2014

*PSEUDOMONAS SYRINGAE* PV. *MACULICOLA 1* (RPM1; Kim et al., 2005; Takemoto and Jones, 2005). RIN4 is phosphorylated upon infection with *P. syringae* expressing either AvrB or AvrRpm1 (Mackey et al., 2002). RIN4 is also involved in the activation of another CNL type R protein *RESISTANCE TO P. SYRINGAE 2* (RPS2) by putative Cys protease AvrRpt2 of *P. syringae*, which causes posttranscriptional cleavage and disappearance of RIN4 and this is required for full RPS2 activation (Axtell and Staskawicz, 2003; Mackey et al., 2003). Interestingly, in coimmunoprecipitation experiments, RIN4 was found to associate with RPM1, RPS2 as well as with pathogen recognition receptor (PRR) FLAGELLIN-SENSITIVE 2 (FLS2), creating thus a physical link between PTI and ETI (Qi et al., 2011). The *rin4* null mutation lethality is rescued in a *rin4rps2* double mutant, indicating that RIN4 negatively regulates inappropriate activation of RPS2 (Mackey et al., 2003). In addition, fragments of RIN4, including those produced by AvrRpt2, each containing a nitrate-induced (NOI) domain specific for plants, suppress PTI, also in the *rpm1/rps2/rin4* mutant background, and activate a cell death response in the wild type (Afzal et al., 2011).

## MEMBRANE TRAFFICKING AND THE R PROTEINS-DEPENDENT IMMUNITY

Surprisingly, several basic regulators expected to function in the endomembrane trafficking and membrane fusion events, such as SNARE and exocyst proteins, might be also connected to the regulation of activity of R proteins. For instance, the dwarf and lesion-mimic double mutant of plasma membrane syntaxins SYP121 and SYP122 constitutively expresses the SA signaling pathway- as well as other known pathogen-responsive genes (Zhang et al., 2008). The same study shows that based on the suppressor mutant analysis of *syp121 syp122*, PAD4 is of key importance for the lesion development. Mutant alleles of signaling mediators of both TNL and CNL-type resistances *EDS1*, *NDR1*, *REQUIRED FOR MLO12 RESISTANCE 1* (*RARI*) and *SUPPRESSOR OF G-TWO ALLELE OF SKP1* (*SGT1b*) partially rescued the lesion-mimic phenotype. Interestingly, the double mutant was crossed to the autophagy *atg7* mutant, however, as there was no effect of this mutation on the appearance of lesions, authors concluded that the autophagy does not play a role in this process (Azevedo et al., 2002; Zhang et al., 2008).

Recently, *exo70B1* loss-of-function mutant was found to develop spontaneous leaf lesions, over-express defense responses genes and show enhanced resistance to fungal, oomycete and bacterial pathogens (Kulich et al., 2013; Stegmann et al., 2013). Unexpectedly, its function is not related to the secretion of secretory vesicles to the plasma membrane; instead, EXO70B1 positive compartments were found to end in the central vacuole and to co-localize with autophagosomal marker ATG8f. In a screen for mutants that suppress *exo70B1* phenotype, nine alleles of TIR-NBS2 (TN2) were identified, suggesting that loss-of-function of EXO70B1 leads to activation of this TN protein (Zhao et al., 2015). It was also shown that TN2 interacts with EXO70B1 in yeast and *in planta*. However, it is not known whether TN2 directly monitors EXO70B1 integrity (as proposed by Zhao et al., 2015) or whether EXO70B1 is only required for autophagic transport to the vacuole and subsequent degradation of TN2. EXO70B1-mediated autophagy-related transport to the vacuole might be participating in TN2 degradation. Both scenarios would explain the observed phenotype.

Additionally, recent work confirmed the importance of membrane trafficking in the plant cell death lesion suppression – *lazarus 4* (*laz4*) was found to be mutated in one of three *VACUOLAR PROTEIN SORTING 35* (*VPS35*) genes which code for a subunit of the retromer complex functioning in endosomal protein sorting and vacuolar trafficking – esp. of retrograde retrieval of vacuolar sorting receptors. These results also showed that the retromer deficiency impairs endosomal sorting of immune components and targeting of vacuolar cargo (Munch et al., 2015).

Interestingly, the endosomal compartment may be as well the site of R-Avr proteins interaction – potato R3A and *Phytophthora infestans* effector AVR3a interact and relocalize from the cytoplasm to endocytotic compartment from where they turn on HR signaling (Engelhardt et al., 2012).

Even though it was not described for plants so far, we can expect that the both endosomes and autophagy related membrane trafficking will provide pathogens an opportunity to manipulate both for the purposes of more successful infection. Such an example was recently described for human epithelium-*Salmonella* interaction – at early stages of *S. typhimurium* infection, autophagy is used to seal endosomal membranes damaged by *Salmonella* secretion system during host cell invasion, but later it is also necessary for the further progression of *Salmonella* infection (Kreibich et al., 2015).

## FROM AUTOPHAGY TO IMMUNITY

Autophagy is a bulk degradation by which cell/organism recycles nutrients, deals with stress, clears off dysfunctional organelles, aggregates etc. (Levine and Klionsky, 2004). Several types of autophagy have been reported, including macroautophagy, which is present in many organisms including fungi, animals and plants. This process relies on the concerted action of autophagy-related (*ATG*) genes encoded proteins to form first phagophore, to promote phagophore enclosure into autophagosome, and to deliver autophagosomes to the vacuole or lysosomes to release the autophagic bodies for eventual breakdown (Li and Vierstra, 2012; Reggiori and Klionsky, 2013).

When *Arabidopsis* mutants are disrupted in *ATG* genes represented by single loci, they grow normally under non-stress conditions, but are hypersensitive to nitrogen and carbon starvation (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005). However, unlike other non-plant organisms, *Arabidopsis* has nine *ATG8* and two *ATG12* gene isoforms, which makes the study of their role more difficult and suggests that the autophagic process in plants is more complicated than in other organisms. Some of its complexity is reflected in the role of autophagy in plant immunity.

The importance of autophagy in the plant immunity was first demonstrated in Liu et al. (2005) – it was found that the autophagy was required to restrict the spread of plant HR cell death. The activation of hypersensitive cell death via the R gene RPM1 upon infection with bacteria also led to cell death beyond the borders of the infection site in plants silenced for *atg6/Beclin1* (Patel and Dinesh-Kumar, 2008). It was concluded that autophagy prevents unrestricted HR cell death and that functions as a pro-survival pathway in plant–pathogen interactions. All of these observations and conclusions were based on experimenting with older *Arabidopsis* plants and on tissues surrounding the actual infection sites, a few days after local infection.

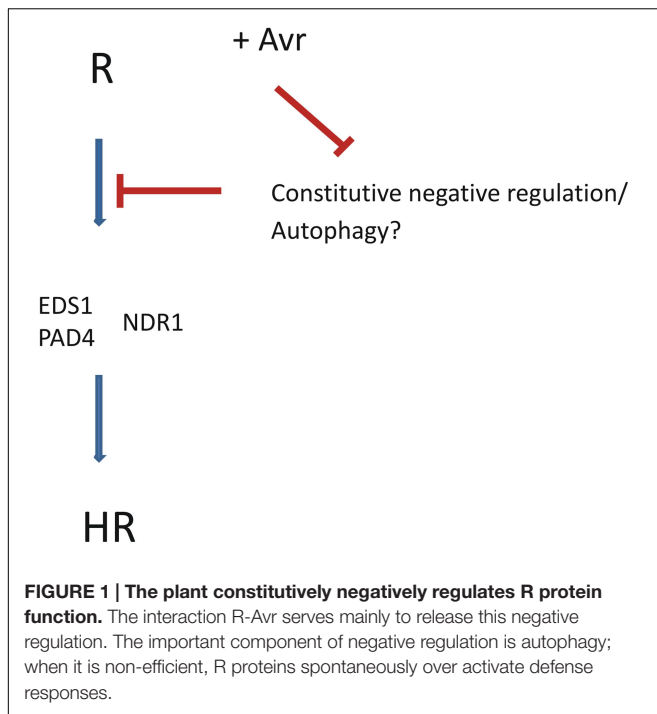
However, a pro-death function of autophagy during HR cell death was reported as well (Hofius et al., 2009). Autophagy was found to be triggered by some, but not all types of R proteins in the infected tissue and its surroundings. HR cell death triggered by R proteins RPS4, RPP1 and RPM1 was significantly suppressed in *atg* (autophagy) mutants; especially the first two of them which signal through EDS1 signaling component. In this case, cell death was monitored in the actual infection site, in the range of hours after inoculation (Hofius et al., 2009).

Yoshimoto et al. (2009), found no deviations in RPM1-triggered cell death beyond the initial infection site in younger *atg* mutants. However, in older *atg* mutants such as *atg5*, they observed lesions in non-infected tissues 6–9 days after infection. Interestingly, these effects were suppressed by removal of the SA and by mutations in SA signaling hub “non-expressor of *PR* genes” – NPR1. The authors proposed that autophagy negatively regulates the cell death by controlling NPR1-dependent SA signaling. In contrast to younger leaves, older *atg* mutant leaves contain higher levels of toxic metabolites, disrupted organelles and oxidized proteins which contribute to the cell death spread (Yoshimoto et al., 2009). This could be as well explained as a

combination of effects of different sets of genes involved in the adult plant resistance and ineffective autophagy (Carviel et al., 2009). Scientists tried to explain and integrate these conflicting results obtained from studies on HR lesions of *atg* mutants. Zhou et al. (2014) propose that autophagy suppresses SA and ROS signaling amplification loop that leads to cell death, while in the resistance to necrotrophic pathogens it promotes JA signaling. Consistently, a recent hypothesis suggests that SA is not only an autophagy inducer, but also a cargo for autophagy-related ER to vacuole membrane transport and catabolism (Kulich and Zarsky, 2014). Recently, a model was worked out in which the autophagy is both initiator and executioner of cell death and is placed downstream of the R protein activation, and supposed to help the cell to deal with the ER stress provoked by a heavy load with pathogenesis related proteins (PRs, review in Minina et al., 2014).

## CONCLUSION AND PERSPECTIVES

Here we show that most of the observed defects in *Arabidopsis* R protein regulator mutants are a direct or indirect consequence of non-pathogen related ectopic R protein activation. It thus seems conceivable that the plant constantly down regulates R protein function, and when this constitutive negative regulation is disturbed, the R proteins are activated and spontaneously signal the non-existent pathogen attack. Based on the example of *rin4* mutant lethality we could speculate, that, similarly to other organisms, the proper function of the negative control might be set already in the earliest stages of development. The plant innate immunity has to be kept as low as possible when it is not necessary in order to prevent high energy costs of defense, and yet in the state of alertness which will allow its fast, in fact instantaneous, activation. We believe that the best way to achieve this is to keep these components (i.e., in our case R genes) transcribed and translated on a sufficient basic level, but to keep their function tightly under negative control which will prevent undesired overactive autoimmunity. How could be this achieved? There are many examples of negative controls involved at various stages of defense that include ubiquitination and proteolysis, phosphorylation of proteins, as well as redox dependent changes in protein multimerization and localization (e.g., Trujillo et al., 2008; Anderson et al., 2011; Vogelmann et al., 2012). We suggest that one of the mechanisms to achieve this is also targeting of defense machinery components – here especially R proteins – to the autophagy pathway for degradation (**Figure 1**). Once the R protein is recruited by autophagy machinery into the autophagosome, it might share the destiny of other autophagic cargos – transport to the vacuole and degradation. We speculate that along with proteins the autophagy related degradation process might destroy also other molecules including signaling relevant molecules as ROS or SA. After the interaction of R protein with its counterpart Avr, R protein is protected against this autophagy-dependent degradation and can interact with downstream components and trigger ETI. This model may be valid also for indirect R-Avr interactions; e.g., the proposed R protein guard function (reviewed e. g. in Spoel and Dong, 2012)



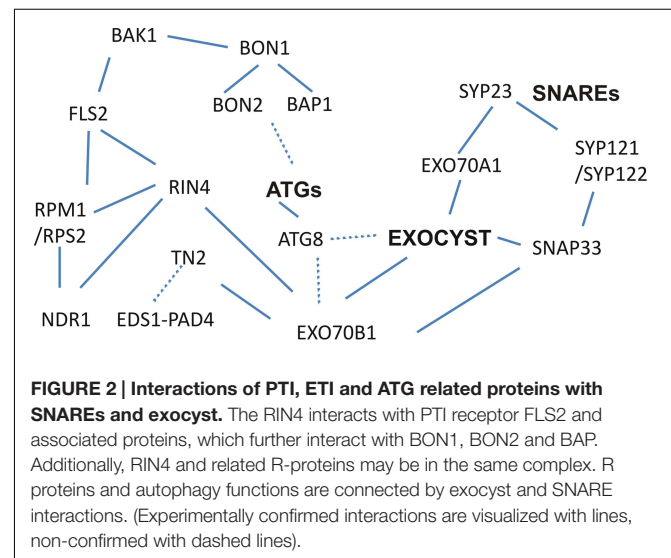
could be based on the avoidance of this negative regulation after the recognition of the changed status of the guardee. It should be stressed that we certainly expect other ways of regulations of R proteins to exist, such as a switch from inactive to active state of R protein upon Avr recognition, as well as other ways of negative regulation.

Based on our hypothesis one would expect that autophagy mutants should copy the phenotype of *exo70B1* mutant, having at least some R proteins constitutively activated. While some of the mutants in the autophagy pathway indeed show similar phenotypic deviations (e.g., early senescence and yellowing, sensitivity to starvation, as well as SA hyperaccumulation in *atg2* and *atg5* mutants; Yoshimoto et al., 2009; Wang et al., 2011), others seem to display only early senescence phenotype and cell death phenotypes only after starvation induction (like *atg7* mutant). It also seems that some subunits of autophagy machinery might be more important for the negative regulation of the immunity, while others, e.g., ATG7 and ATG9, in the execution of HR (Hofius et al., 2009; Minina et al., 2014). It should be, however, noted that autophagy proteins (and EXO70B1) have been also implicated in diverse cellular processes independently of their roles in autophagy.

We also expect that, pathogen effectors might have evolved to manipulate and hijack this negative regulation and worsen the plant defense – recently, a *Phytophthora infestans* effector PexRD54 has been shown to outcompete the autophagy cargo receptor Joka and enhance virulence of this pathogen. Interestingly, PexRD54 does this probably through the activation of selective autophagy. Joka could participate in the removal of plant or pathogen molecules that negatively affect host defenses. As authors of the study speculate, PexRD54 would thus counteract the positive role of Joka2-mediated selective

autophagy in pathogen defense. An alternative, but not exclusive explanation based on our hypothesis would be that PexRD54 at the same time stimulates the selective autophagy of R proteins capable of detecting it and thus promotes pathogen virulence (Dagdas et al., 2016). Already the report of Engelhardt et al. (2012) demonstrated the capability of cytoplasmic R protein to be recruited to endomembranes, but not for degradation, rather for the purpose of activation. However, this is not exclusive with our model – the interaction of R3A and Avr3A might release the negative regulation of R3A and switch on the HR. This interaction is obviously indirect and requires an intermediate connected to ARA6/ARA7 marked endosomes. It is possible that this activation evolved from the mechanisms of negative regulation. More information on R3A and Avr3A interactors could help to solve this ambivalent situation.

We found an indirect support for our hypothesis in the autophagy-related events described for mammalian cells – it is known from experiments performed on HeLa cells that endocytosed plasma membrane contributes to ATG12–ATG5–ATG16L1-positive/ATG8-negative phagophore precursor vesicles by both clathrin-dependent and -independent routes (Moreau and Rubinsztein, 2012). The subsequent maturation of these small phagophore precursors into phagophores (ATG12–ATG5–ATG16L1-positive/ATG8-positive) is assisted by SNARE-mediated homotypic fusion that increases their size. Additionally, *Arabidopsis* BON1/2/3 belong to copine proteins, a family of ubiquitous Ca(2+)-dependent, phospholipid-binding proteins that are known to be involved in animal membrane trafficking events (Tomsig and Creutz, 2002), and in *Dictyostelium* localize to plasma membrane, contractile vacuoles, organelles of the endolysosomal pathway, and phagosomes (Damer et al., 2005). Therefore, besides confirmed role of EXO70B1 in autophagy and regulation of TN2 activity, very probably SNARE and BON proteins could implement similar role in autophagy-related membrane targeting and membrane fusion events leading to the negative control of R proteins.



We found further support for this hypothesis in the connection between assumed autophagy regulating proteins and R proteins, as well as other key molecules of the both PTI and ETI immune response, in the web of protein-protein interactions that are available in Biogrid and PPIN databases (Stark et al., 2006; Mukhtar et al., 2011; **Figure 2; Table 1**). The components of PAMP-sensing complexes interact with RIN4, which further interacts with R proteins. Mainly through mediating kinase BAK1, they are connected and interact as well with BON1, BON2 and BAP. RIN4 interacts with R proteins as well as with EXO70B1 (Afzal et al., 2013). Besides its capability to interact with other exocyst and SNARE proteins, EXO70B1, together with 20 other paralogs of *Arabidopsis* EXO70 exocyst subunits, possess ATG8 interacting motives, which indicates that the autophagy machinery and exocyst complex functions are multiply connected (Cvrčková and Zárský, 2013; Tzfadia and Galili, 2013; Sabol et al., in preparation). Thus, in the vicinity of plasma membrane, and depending on membrane trafficking which involves SNARE, exocyst and autophagy complex proteins, a tight control of R protein activation allows the immunity to be kept low but in a constant alert.

Recently, a role for EXO70F3 of *Oryza sativa* in immunity against *Magnaporthe oryzae* was found – OsEXO70F3 appears to play a crucial role in immunity triggered by Pii, suggesting a role for this EXO70 paralog as a decoy or helper in Pii/Avr-Pii interaction (Fujisaki et al., 2015). It may be true that pathogen effectors target these and other exocyst subunits in order to suppress defense, however, we don't consider it to be mutually exclusive with our hypothesis.

Our model could help to better understand and reconcile conflicting aspects of autophagy in the plant immunity (Teh and Hofius, 2014): in the infection sites, R-Avr recognition prevents R protein targeting to inactivation/destruction pathway and triggers the ETI, and with the increased distance from the infection site, declining concentration of Avr protein allows the autophagy to overtake again a control over R protein. In *atg* mutants, the existing constitutive immunity activation results in spontaneous HR lesions formation; but after the pathogen attack, in the case of younger leaves, in addition to R protein deregulation, R is further activated by Avr recognition, which makes cells more resistant and lesions smaller. Or, under conditions with additional stresses, as in the case also of older leaves, because of coincidence between consequences of ineffective autophagy of *atg* mutants and Avr-enhanced over activation of R proteins, less Avr is needed for HR threshold to be crossed and lesions spread farther.

Our model's aim is to focus on one aspect only – a possibility of a negative regulation of some NLRs/innate immunity related proteins by autophagy in plants. However, there are many difficulties that will have to be overcome in order to confirm

its validity. Part of difficulties is coming from the complexity of autophagy machinery and a large number of ATG proteins that have also been implicated in diverse cellular processes independently of their roles in autophagy. Autophagy machinery is also difficult to study separately from other endomembrane compartments, especially by using pharmacological treatments. For instance, wortmannin, which is often used for these purposes, is rather pleiotropic drug – dependent on cell type and concentration it affects different types of phosphoinositide kinases, having thus multiple interference with endomembrane dynamics.

To conclude, plants have mechanisms to downregulate R proteins function, and when they are attacked by an appropriate Avr carrying pathogen, the R proteins are stabilized, activating defense responses. This would also mean that R proteins are capable of immunity activation without Avr and that the interaction R-Avr serves mainly to release R proteins negative regulation. The disturbance of the basic autophagy machinery has pleiotropic effects on many plant functions including development and is influenced by growth conditions, abiotic stresses and senescence, hence it is very difficult to study effects of *atg* mutants that would concern specifically defense responses. We believe that in the near future, R proteins studies will bring more information on the regulation of their activity including proteins that target them for the suggested autophagy destruction.

## AUTHOR CONTRIBUTIONS

TP did a compilation of data on R-related dwarf and lesion mimic mutants and most of the writing; PS did the RIN4-related data mining and text editing; IK explained the connection to autophagy; JO dealt with membrane trafficking chapter; VZ did the most of text editing and integrating as well as the finalization of the manuscript.

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