Phytophthora infestans RXLR effector AVR1 and its host target Sec5

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Chapter 1

General introduction

Introduction

At this very moment nearly 870 million people are undernourished in the world (FAO annual report 2013). How to increase the food production on the available land to feed the growing human population is a global question. Controlling losses due to plant pathogens is essential to increase food production. Pesticide spraying used to be the most popular and efficient way to control plant pathogens. However, it causes environmental problems and renders pathogens to build up tolerance towards the chemicals used. Thus, development of efficient, sustainable, and environmental friendly protection methods is of great need to modern agriculture. Understanding the mechanisms how pathogens colonize their host plants and how plants are defending themselves against intruders helps us to acquire more durable resistant plants and new control methods.

The aim of the research described in this thesis is to gain more insight into molecular processes that govern the interaction between the late blight pathogen *Phytophthora infestans* and its host plants. In **this Chapter**, I will first introduce the late blight pathogen and describe the basics of plant defense responses. Subsequently, I will touch upon effectors secreted by pathogens and give some insight in the mechanisms that effectors may exploit to manipulate host defense.

Phytophthora infestans, the causal agent of potato late blight

Potato is the third most important food crop in the world. It provides high yields and nutritious food and as such is a major and important food resource, especially for developing countries. Without pest control about 75% of the estimated potato yield would be lost (Oerke, 2006). A major potato pathogen is the oomycete *P. infestans* which causes the notorious late blight disease. This infamous plant pathogen was the indirect cause of the Irish potato famine in the 1840s, and even nowadays *P. infestans* is responsible for billions of dollars of losses every year to modern agriculture (Fry, 2008). Effective late blight disease management requires costly chemical spraying, which puts pressure on the environment. *Phytophthora* literally means plant destroyer; the genus includes over 100 recognized species that are all plant pathogens (Kroon et al., 2012). *Phytophthora capsici*, *Phytophthora sojae*, *Phytophthora parasitica* and *P. infestans* are a few well-studied species in the *Phytophthora* genus. *Phytophthora* species generally cause foliage blight or stem and root rot as well as lesions on stems and fruits on a broad range of plants, including pepper, cucumber, eggplant, melon, soybean, tomato and potato (Kroon et al., 2012).

Infection by *P. infestans* generally starts via windblown sporangia that land on a host leaf surface and release zoospores. Zoospores encyst, germinate, and form appressoria to penetrate epidermal host cells. After penetration, hyphae grow into the mesophyll where they proliferate intracellularly and form feeding structures called haustoria. In a later stage, the infected tissue necrotizes and hyphae develop sporangiophores that escape the leaf via stomata, and produce numerous sporangia.

P. infestans has the largest genome size of all oomycetes sequenced so far. The genome size is 240 Mb, of which 74% is repetitive DNA (Haas et al., 2009; Quinn et al., 2013). The genome of *P. infestans* has a bipartite structure with the core orthologs mainly located in gene dense regions, and effector genes located in gene sparse and repeat-rich regions. The latter are dynamic regions and provide plasticity to the *P. infestans* genome, thus promoting rapid evolution of effector genes. As a result *P. infestans* has a variable repertoire of effector genes (Haas et al., 2009) and is able to adapt rapidly to overcome late blight resistance in potato introduced by breeding (McDonald and Linde, 2002). Consequently, most of the nowadays used potato cultivars are susceptible to *P. infestans*.

Two layers of plant defense response

To defend against pathogen invasion, plants have developed two layers of defense, which is conferred by two different groups of immune receptors. The first layer of plant defense is mediated by plasma membrane-associated receptors known as pattern recognition receptors (PRRs), which are homologous to animal Toll-like receptors. These are Receptor-Like Kinases (RLKs) that consist of an extracellular domain, a membrane spanning domain and an intracellular kinase domain. Another class comprises Receptor-Like Proteins (RLPs) that, compared to RLKs, lack the kinase domain. PRRs perceive pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) and trigger PAMP-triggered immunity (PTI). The best known plant PRRs are the RLKs FLS2 and EFR that recognize bacterial ligands. The Arabidopsis PRR FLS2 recognizes bacterial flagellin and activates PTI that restricts the growth of *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 (Zipfel et al., 2004; Bauer et al., 2001). FLS2 is wide spread in plants. It is present in Arabidopsis, tomato, *Nicotiana benthamiana* and rice. In contrast, EFR, which recognizes the bacterial elongation factor Tu (EF-Tu), is restricted to *Brassicaceae* (Monaghan and Zipfel, 2012).

Well known PRRs that confer resistance against fungal pathogens are so far only RLPs, such as tomato Eix1 and Eix2 that are known to bind fungal xylanase (Ron and Avni, 2004). The tomato Ve1 is a RLP that confers resistance towards the vascular fungal pathogen *Verticillium dahliae* by recognizing the effector Ave1 (de Jonge et al., 2012). The Arabidopsis lectin receptor kinase LecRK-I.9 is a RLK-type PRR that acts as a resistance component against oomycete pathogens. It was found to bind with the RGD cell attachment motif in the *P. infestans* RXLR effector IPI-O (Bouwmeester et al., 2011). Another PRR active against *P. infestans* was identified recently. It is known as ELR, which recognizes elicitin proteins from several *Phytophthora* species (Du, 2014).

The second layer of plant defense is mostly mediated by a group of intracellular receptors, which are nucleotide-binding leucine-rich repeat (NLR) proteins also known as resistance (R) proteins. They consist of an N-terminal domain that is either a coiled-coil domain or a toll/interleukin 1 receptor domain, followed by a conserved nucleotide-binding domain and a C-terminal leucine rich repeat domain (Takken and Tameling, 2009). NLRs are found in a wide spectrum of plant species and are able to confer resistance against different plant pathogens, including fungi, oomycetes, bacteria, viruses and nematodes (Dangl and Jones, 2001; Hulbert et al., 2001). So far, all the resistance genes that function against *P. infestans* encode NLRs. Plant NLRs can recognize their matching effectors and activate effector-triggered immunity (ETI). Compared to PTI, ETI is generally a faster and stronger immune response that is largely associated with a programmed cell death known as the hypersensitive response (HR) (Dangl and Jones, 2006). As is described in detail in **BOX 1**, it is generally believed that NLR proteins have nuclear functions.

The way pathogen effectors are detected by their corresponding NLR proteins can be either direct through physical association or indirect through interaction via another host protein. Examples pointing to direct physical association between a pathogen effector and its corresponding R protein, as demonstrated by Y2H assays, include the Avr-pita effector of the rice blast pathogen *Magnaporthe oryzae* with the rice R protein Pi-ta, the *M. oryzae* effector AvrCO39 with the rice R protein RGA4/RGA5, the bacterial effector PopP2 from *Ralstonia solanacearum* with the Arabidopsis R protein RRS1-R, and tobacco mosaic virus (TMV) helicase domain P50 with its resistance protein N (Jia et al., 2000; Cesari et al., 2013; Deslandes et al., 2003; Ueda et al., 2006). Indirect interactions have been reported for effectors from the bacterial pathogen *P.*

syringae. AvrRpt2, AvrRPM1 and AvrB were found to manipulate Arabidopsis RIN4 to increase plant susceptibility but when RIN4 is guarded by the NLRs Rpt2 and RPM1, immunity is induced. AvrPphB is able to process the host protein kinase PBS1 and this cleavage results in activation of PBS5-mediated resistance (Axtell et al., 2003; Mackey et al., 2003; Shao et al., 2003). So far, only two examples of indirect interaction have been reported for filamentous plant pathogen effectors. These are Avr2 from *Cladosporium fulvum*, which is recognized by tomato Cf-2 via the host cysteine protease RCR3 (Rooney et al., 2005), and the RXLR effector AVR2 from *P. infestans*, which is perceived by potato R2 via the host protein BSL1 (Saunders et al., 2012). It has been hypothesized that indirect interaction enables R proteins to recognize effectors from a broad range of pathogens by guarding effector targets (Dangl and Jones, 2001). The latter are host proteins targeted by pathogen effectors and also referred to as host targets.

Previously PTI and ETI were considered to be two distinct immune response pathways (Dangl and Jones, 2001). However, based on an increasing number of studies it has been hypothesized that there is no clear distinction between PTI and ETI (Thomma et al., 2011). PTI is conferred by a group of extracellular receptors known as PRRs, whereas ETI is conferred by intracellular receptors and in most cases by NLR proteins. PTI is normally broad-spectrum and not associated with cell death while ETI is strain specific and often associated with a fast and robust HR. PTI is a weak immune response which reduces pathogen colonization. In contrast, ETI is a strong immune response and totally hampers pathogen invasion. However, recent studies that more closely investigated and compared the downstream signaling of both immune responses, have shown that the downstream signaling cascades are largely overlapping (Mur et al., 2008). Besides that, ETI can be broad-spectrum, e.g. the tomato RLP Cf4 recognizes *Cladosporium fulvum* effector AVR4 in a race specific manner, but it also recognizes AVR4 homologs from other pathogenic fungi (Stergiopoulos et al., 2010). Not only ETI can trigger HR, PTI can trigger HR as well. Naito et al. (2007) for example, showed that flg22 induces HR in Arabidopsis. The strength of immunity and HR are also not necessarily weaker in a PTI response. The tomato RLP Ve1 recognizes the effector Ave1 from *V. dahliae* and confers full resistance to this pathogen, which resembles ETI (de Jonge et al., 2012). However, Ave1 has homologs in several fungi, the bacterium *Xanthomonas axonopodis* and even in plants and thus in combination with the RLP nature of Ve1, the immune response induced by Ave1 has the characteristics of PTI.

BOX 1: Subcellular targeting of NLRs and their corresponding avirulence effectors.

In Arabidopsis 72% of the NLR proteins were predicted *in silico* to localize in the nucleus as they contain a nuclear localization signal (NLS) (Meyers et al., 2003). However, several studies have shown that NLRs are not restricted to the nucleus. Some NLRs are localized on the plasma membrane or have a nucleocytoplasmic distribution. It is generally accepted that NLRs have nuclear functions because they are known to trigger nuclear-associated immunity (Shen and Schulze-Lefert, 2007; Shen et al., 2007; Elmore et al., 2011). For some NLRs a nucleocytoplasmic distribution is also shown to be important for NLR-mediated immunity. For example, potato NLR Rx needs a certain nucleocytoplasmic distribution to confer resistance to potato virus X (PVX), and N protein which is likely to perceive its corresponding TMV effector P50 in the cytoplasm, is translocated to the nucleus for immune signalling (Tameling and Baulcombe, 2007; Burch-Smith et al., 2007). The table below, summarizes what is currently known about the subcellular localization of NLR proteins and how this relates to activation of defense and the subcellular localization of their corresponding effectors.

Subcellular localization of NLRs and their corresponding avirulence effectors.

NLR	Plant	Subcellular localization	Defence related location	Effector	Pathogen	Effector localization	Avirulence related location	Ref.
I-2	tomato	-	-	Avr2	*Fusarium oxysporum*	N & C	N	1
MLA10	barley	N & C	N	A10	*Blumeria graminis*	-	-	2
Rx	potato	N & C	N & C	CP	potato virus X	-	-	3
N	tobacco	N & C	N	P50	tobacco mosaic virus	N & C	N & C	4
RPM1	Arabidopsis	PM	PM	AvrRpm1 and AvrB	*Pseudomonas syringae* pv.*maculicola*	PM	PM	5
RPS4	Arabidopsis	N & C	N	AvrRPS4	*Pseudomonas syringae*	N & C	N	6,7
RRS1-R	Arabidopsis	N	-	PopP2	*Ralstonia solanacearum*	N	N	8
SNC1	Arabidopsis	N & C	N	-	-	-	-	9

- = not determined; N = nucleus; C = cytoplasm; PM = plasma membrane; 1, Ma et al. (2013); 2, Shen et al. (2007); 3, Tameling and Baulcombe (2007); Slootweg et al. (2010); 4, Burch-Smith et al. (2007); 5, Gao et al. (2011); 6, Wirthmueller et al. (2007); 7, Heidrich et al. (2011); 8, Bernoux et al. (2008); 9, Cheng et al. (2009).

ETI is often associated with a hypersensitive response (HR), which is thought to hamper pathogen infection. However, studies that artificially target NLRs or their corresponding effectors to either the nucleus or cytoplasm by using the nuclear localization signal (NLS) and nuclear export signal (NES) have shown that HR and resistance are bifurcated. When the barley NLR MLA10 is targeted only to the nucleus, it is sufficient to confer resistance against *Blumeria graminis* but not able to trigger HR. However, MLA10 triggers an enhanced HR when it is artificially targeted to the cytoplasm in *N. benthamiana*, but loses the ability to confer resistance (Bai et al., 2012; Chang et al., 2013). A study on the *Pseudomonas syringae* type III effector AvrRps4 has shown that the nuclear targeted AvrRps4 could trigger its corresponding NLR Rps4 to mediate resistance but failed to trigger HR (Heidrich et al., 2011). These results indicate that NLR-mediated resistance and HR can occur in different subcellular compartments.

Effectors secreted by *P. infestans*

The effectors secreted by *P. infestans* can be roughly divided into two classes according to the place where they exert their functions, namely extracellular and intracellular effectors. The extracellular effector class comprises for example, enzyme inhibitors, RGD motif containing proteins, elicitins and NEP-like proteins (NLPs) (Stassen and van den Ackerveken, 2011), which either inhibit host hydrolytic enzymes, interfere with plant cell wall-plasma membrane adhesions or trigger host cell death to modulate the conditions for pathogen invasion. The intracellular effector class comprises RXLR effectors and Crinklers (CRNs), which contain N-terminal motifs required for translocation to host cells.

P. infestans has about 560 predicted RXLR effector genes (Haas et al., 2009). The RXLR effectors are named after a conserved RXLR motif (arginine (R), any amino acid (X), leucine (L), arginine (R)) in the N-terminus and they are only found in oomycete plant pathogens. This RXLR motif, which was first identified in 2005, is a common motif among all the known oomycete avirulence proteins (Rehmany et al., 2005). RXLR effectors contain an N-terminal signal peptide followed by a RXLR motif, which is required for host translocation (Whisson et al., 2007). The C-termini of RXLR effectors show no sequence homology with known proteins and are known as effector domains because they are responsible for effector functions (Bos et al., 2006; Oh et al., 2009; Dou et al., 2008; Champouret et al., 2009; van Poppel et al., 2008). Although the effector domains of RXLR effectors are highly diverse, there are three conserved motifs identified which are known as W, Y and L motifs (Jiang et al., 2008). These motifs were identified in a bioinformatic comparison of all the RXLR effectors encoded in the genomes of *P. sojae* and *Phytophthora ramorum*. More than half of the investigated RXLR effectors contain these motifs, which are about 21 to 30 amino acid residues long and are named after the amino acid W, Y or L that is conserved at a certain position in these motifs. The subcellular localization of RXLR effectors has been studied *in planta* by transient expression of fluorescent tagged effector proteins in leaves of *N. benthamiana*. A study on the subcellular localization of 49 RXLR effectors from the downy mildew *Hyaloperonospora arabidopsidis* showed that they localize to various subcellular compartments in the host cell (Caillaud et al., 2012). The subcellular localization of a RXLR effector is relevant for its biological function. *P. sojae* Avh241 localizes on the plasma membrane, which is critical for triggering plant cell death (Yu et al., 2012). *P. infestans* AVR2 localizes in the nucleus, cytosol and plasma membrane, while a membrane localization is

required to trigger an HR (Saunders et al., 2012). *P. infestans* AVRblb2 was localized at the plasma membrane where it targets and inhibits the host papain-like cysteine protease C14 from secretion to the haustorial interface to promote infection (Bozkurt et al., 2011).

The second class of oomycete intracellular effectors comprises Crinklers (CRN), a large and complex family of effectors. Thus far, *CRN* genes are present in all the sequenced *Phytophthora* genomes but the numbers vary (Haas et al., 2009). Crinklers all share a conserved N-terminal LXLFLAL-motif followed by a DWL motif, with the LXLFLAL-motif being required for host translocation (Schornack et al., 2010). This was shown by fusing the N-terminal part of CRN2, CRN8 and CRN16 to the effector domain of AVR3a and transforming those constructs into *P. capsici*. The *P. capsici* transformants were then inoculated on the *N. benthamiana* plants containing the R protein R3a as transgene. In all three cases AVR3a was successfully delivered into the host as shown by activation of R3a-mediated disease resistance (Schornack et al., 2010). The first CRNs, CRN1 and CRN2, were identified in an *in planta* expression assay to screen for secreted proteins from *P. infestans* (Torto et al., 2003), and were found to trigger necrosis in *N. benthamiana* and tomato plants. Some of the CRNs were shown to function inside the host nucleus, i.e. CRN8 and CRN2 were found to be nuclear localized and both trigger necrosis in *N. benthamiana* (Schornack et al., 2010; van Damme et al., 2012). However, not all CRNs trigger necrosis. Stam et al. (2013) analysed 11 CRNs from *P. capsici* and found that only 3 of these trigger necrosis in *N. benthamiana*. CRN8 has kinase activity and is able to promote *P. infestans* colonization (van Damme et al., 2012). Some CRNs including 83_152 from *P. capsici* (Stam et al., 2013) and PsCRN63 and PsCRN115 from *P. sojae*, were found to contribute to pathogen colonization (Liu et al., 2011). These results indicate that CRNs, like RXLR effectors, contribute to pathogen virulence by manipulating host defense responses. However, there are still many gaps in our knowledge with respect to the downstream signalling of CRN-triggered necrosis and the mechanisms of CRN-facilitated pathogen infection.

Effector-modulated host defense response

In order to colonize the host plant, pathogens secrete multiple effector proteins to modulate host immunity, for example suppressing PTI. The well-studied bacterial type III effectors (T3Es) are able to suppress host immune responses via a variety of strategies. T3Es can target the upstream PTI response by interfering with PRR

signalling, e.g. *P. syringae* AvrPto inhibits RLK activity and AvrPtoB ubiquitinates and subsequently degrades RLKs (Xiang et al., 2008; Gimenez-Ibanez et al., 2009; Goehre et al., 2008). On the other hand, T3Es are able to target PTI downstream signalling like MAPK pathways, e.g. HopF2 inhibits MKK5 preventing MPK3 and MPK6 phosphorylation (Wang et al., 2010), and HopAI1 deactivates and dephosphorylates MPK3, MPK6 and other MAPKs (Li et al., 2007; Zhang et al., 2007). The transcription activator-like T3Es (TALEs) from the plant pathogenic bacterium *Xanthomonas* spp. can act as transcription factors to directly modulate gene expression (Bogdanove et al., 2010), which shows that T3Es can modulate gene expression. T3Es can also alter plant organelle functions, for example HopI1 targets the chloroplast to reduce SA accumulation (Jelenska et al., 2007), and HopG1 alters basal levels of reactive oxygen species in mitochondria (Jelenska et al., 2010). Last but not least, T3Es are able to manipulate host vesicle trafficking, e.g. the *P. syringae* HopM1 can block host vesicle trafficking by destabilizing MIN7, a key factor involved in vesicle trafficking, to prevent the recycling of immunity-related plasma membrane receptors (Nomura et al., 2006; Nomura et al., 2011). As described in detail in **BOX 2** and depicted in **Figure 1**, vesicle trafficking plays an important role in plant defense.

Like bacterial T3Es, *Phytophthora* RXLR effectors have developed multiple strategies to suppress host immunity (**Table 1**). Several RXLR effectors have been shown to be able to suppress host PTI by targeting the MAPK pathway. For example, RXLR effector Avh331 promotes *P. sojae* colonization by suppressing the expression of certain marker genes downstream of MAPK signalling (Cheng et al., 2012), and *P. infestans* PexRD2 interacts with the kinase domain of MAPKKKε to suppress host immunity (King et al., 2014). Zheng et al. (2014) have checked 31 RXLR effectors from *P. infestans* for their role in inhibiting flg22-induced reporter gene activation in tomato protoplasts, and their results show that eight of the analysed RXLR effectors are able to suppress MAP kinase signalling (Zheng et al., 2014). RXLR effectors can also suppress host defense responses by targeting host E3 ubiquitin ligases as shown for *P. infestans* AVR3a that targets and stabilizes the host E3-ligase CMPG1 to suppress plant immunity (Bos et al., 2010). Another RXLR effector named *P. infestans* IPI-O1, was shown to disrupt the adhesion between the host plasma membrane and the cell wall by targeting the host lectin receptor kinase LecRK-I.9 (Bouwmeester et al., 2011). Moreover RXLR effectors can modulate host transcription by targeting transcription factors. This was shown for the RXLR effector PITG_03192 that promotes *P. infestans* colonization by

preventing re-localization of two plant NAC transcription factors from the ER to the nucleus (McLellan et al., 2013). In addition, RXLR effectors are able to suppress host defense by interfering with host focal secretion. *P. infestans* RXLR effector AVRblb2 promotes pathogen colonization via interaction with a host defense related and papain-like cysteine protease C14, and this interaction inhibits the secretion of C14 to the apoplast (Bozkurt et al., 2011). Last but not least, *P. infestans* RXLR effector AVR1 interacts with the host exocyst component protein Sec5 to modulate host vesicle trafficking and promote pathogen proliferation in the host plant (this thesis). The current knowledge on the role of the exocyst and the exocyst subunits in plant development and plant defense is presented in **BOX 2**.

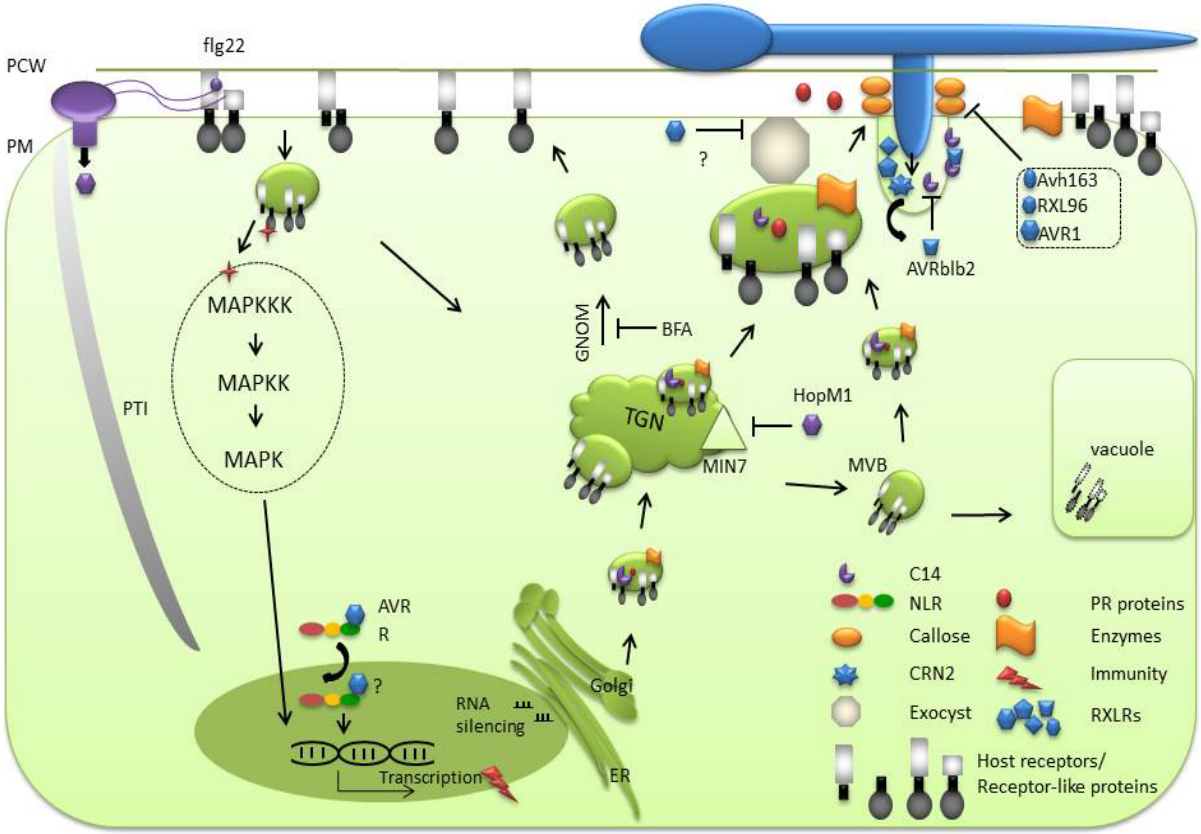

Figure 1. Overview of the role of vesicle trafficking in plant defense and effector-modulated vesicle trafficking. Vesicle trafficking plays a role in host defense against pathogen invasion in two directions, either via endocytosis of PRRs or exocytosis of antimicrobial compounds towards the infection sites. Plant pathogens secrete effector proteins that target proteins that are essential for vesicle trafficking and in this way modulate host defense.

BOX 2: Vesicle trafficking and the exocyst in relation with plant defense responses

Vesicle trafficking plays a role in plant defense against pathogen invasion in two ways (Figure 1). The first is via endocytosis of the ligand-stimulated PRRs upon ligand perception, e.g. flg22 is able to induce the endocytosis of the flagellin receptor FLS2 to activate downstream signalling (Robatzek et al., 2006). The other way is via secreting antimicrobial compounds to pathogen infection sites to either kill the pathogen or reinforce the cell wall of the plant to form physical barriers (Robatzek, 2007). Genetic studies on the role of SNARE proteins in plant defense have shown that barley PEN1, HvROR2 and HvSNAP34 are required for cell wall resistance (Collins et al., 2003). The *Nicotiana benthamiana* syntaxin SYP132 is required for plant defense against bacteria and for PR-1 secretion (Kalde et al., 2007). The Arabidopsis MIN7 protein is a key regulator in vesicle trafficking that plays a role in plant defense against the bacterium *P. syringae* (Nomura et al., 2006). The vesicle-associated membrane proteins VAMP721/722 are directed and moved to the penetration site of powdery mildew *Blumeria graminis,* and are required for disease resistance against *B. graminis* (Kwon et al., 2008). Thus the PEN1-SNAP-VAMP721/722 complex is suggested to be involved in focal secretion.

One step upstream of SNAREs is the exocyst, which is a protein complex of eight subunits that tethers the secretory vesicles to the plasma membrane (He and Guo, 2009). The exocyst is known to play an essential role in plant development as it is involved in exocytosis (Synek et al., 2006). However, there are still many open questions about the role of this protein complex. For example which types of exocytosis is the exocyst complex involved in? What kind of proteins are dependent on the exocyst for secretion? And is the exocyst also involved in cellular process other than basic vesicle trafficking? Few recent studies pointed to the role of the exocyst in plant defense, i.e. in Arabidopsis Exo70H1 and Exo70B2 are important for cell wall-associated defense and Exo70B2 is also required for PTI (Pecenkova et al., 2011; Stegmann et al., 2012). In barley an Exo70F-like gene is found to be important for plant penetration resistance against the fungal pathogen *Blumeria graminis* f.sp. *hordei* (Ostertag et al., 2013). The table below summarizes what is known about the role of exocyst subunits in plant development and defense responses. It is obvious that our current understanding of the exocyst is still rather limited and that there is a need for further investigation of the role of exocyst components in plant defense.

Exocyst component	Function	Reference
Sec3	essential for embryo development	Zhang et al., 2013
Sec5	pollen germination and pollen tube growth	Hala et al., 2008
Sec6	pollen germination and pollen tube growth; involved in cytokinesis and cell plate maturation	Hala et al., 2008; Fendrych et al., 2010
Sec8	contributes to PIN auxin efflux carrier recycling and polar auxin transport; pollen germination and pollen tube growth; involved in cytokinesis and cell plate maturation	Drdova et al., 2013; Hala et al., 2008; Fendrych et al., 2010
Sec15a	pollen germination and pollen tube growth	Hala et al., 2008
Sec15b	involved in cytokinesis and cell plate maturation	Fendrych et al., 2010
Exo70A1	contributes to PIN auxin efflux carrier recycling and polar auxin transport; involved in cytokinesis and cell plate maturation	Drdova et al., 2013; Fendrych et al., 2010
Exo70B2	target of the Ubiquitin Ligase PUB22; negative regulator of PTI; necessary for immune responses against different pathogens; involved in the response to the pathogens *Blumeria graminis* and *Pseudomonas syringae*	Stegmann et al., 2012; Pecenkova et al., 2011
Exo70F*	essential for host penetration resistance to *B. graminis*	Ostertag et al., 2013
Exo70H	involved in defence responses to *B. graminis* and *P. syringae*	Pecenkova et al., 2011
Exo84b	involved in cytokinesis and cell plate maturation	Fendrych et al., 2010

*, All these studies are perfomed on the exocyst subunits in Arabidopsis with the exception of EXO70F, which is studied in barley.

Table 1. Oomycete RXLR effectors with described virulence functions and their host targets.

Effector	Species	Host target	Effector modulated cellular process	Reference
AVR1	*P. infestans*	Sec5	Associates with Sec5 to modulate exocytosis	this thesis
AVR2	*P. infestans*	BSL1	Associates with BSL1 and R2, and induces ETI	Saunders et al., 2012
AVRblb1 (IPI-O1)	*P. infestans*	LecRK-I.9	Interferes with CW-PM adhesion	Bouwmeester et al., 2011
IPI-O4	*P. infestans*	unknown	Supresses ETI triggered by IPI-O1/Rpiblb1	Chen et al., 2012
AVRblb2	*P. infestans*	C14	Prevents secretion of plant protease C14	Bozkurt et al., 2011
Pi03192	*P. infestans*	NTP1/NTP2	Prevents nuclear localization of NAC TFs	McLellan et al., 2013
AVR3a	*P. infestans*	CMPG1	Stabilizes CMPG1 to prevent host cell death	Bos et al., 2010
PexRD2	*P. infestans*	MAPKKKε	Suppresses plant immune signaling	King et al., 2014
AVR3b	*P. sojae*	unknown	Suppresses plant cell death by acting as an ADP-ribose/NADH pyrophosphorylase	Dong et al., 2011
PSR1/PSR2	*P. sojae*	unknown	Suppresses host plant RNA silencing	Qiao et al., 2013
Avh331	*P. sojae*	unknown	Modulates host MAPK signaling	Cheng et al., 2012
PsAvh163	*P. sojae*	unknown	Suppresses callose depositon	Anderson et al., 2012
AVR1b-1	*P. sojae*	unknown	Suppresses BAX-triggered cell death	Dou et al., 2008
PSE1	*P. parasitica*	unknown	Modulates host auxin physiology	Evangelisti et al., 2013
HaRXL96	*H. arabidopsidis*	unknown	Suppresses cell death and callose depositon	Anderson et al., 2012
ATR1	*H. arabidopsidis*	unknown	Suppresses PTI	Sohn et al., 2007
ATR13	*H. arabidopsidis*	unknown	Suppresses PTI	Sohn et al., 2007

Scope of this thesis

AVR1 is a RXLR effector secreted by *P. infestans* that is recognized by the potato NLR protein R1 and triggers R1-mediated resistance. The research described in **this thesis** deals with the *P. infestans* RXLR effector AVR1, the matching R protein R1 and the AVR1 host target Sec5. AVR1 is a typical RXLR effector composed of a signal peptide, an RXLR motif and a C-terminal domain that contains two W motifs and one Y motif. The *AVR1* gene is present in *P. infestans* isolates that are recognized by potato lines containing the *R1* gene. Isolates that are not recognized contain a homolog of the *AVR1* gene that we named *AVR1-like*. The *AVR1-like* gene is located at a different locus than *AVR1*. It encodes a RXLR effector gene that shares 82% identity with AVR1 at the amino acid level, and similar to AVR1, AVR1-like comprises a signal peptide, an RXLR domain and a C-terminal domain with two W motifs and one Y motif.

In **Chapter 2** of this thesis we investigated which parts of AVR1 are required for triggering R1-mediated HR. Motif swap or deletion constructs of AVR1 and AVR1-like were made and co-agroinfiltrated with R1 into leaves of *N. benthamiana* to analyse which motif is essential for R1-recognition.

In **Chapter 3** of this thesis the subcellular localization of R1 was studied. Since both R1 and AVR1 are nucleocytoplasmic proteins, the subcellular compartment that is required for R1 perception of AVR1 and R1-mediated immune activation was analysed. Both AVR1 and R1 were fused with a nuclear localization signal or nuclear export signal and analysed for their ability to trigger HR.

Chapter 4 is focussed at understanding the mechanisms that the *P. infestans* RXLR effector AVR1 uses to modulate host defense. The exocyst component Sec5 was identified as a host target of AVR1 by yeast-two hybrid assays, and the function of Sec5 in host defense was investigated. The *in planta* association of AVR1 and its host target was confirmed by co-immunoprecipitation and bimolecular fluorescent complementation (BiFC).

In **Chapter 5** the potential virulence activities of AVR1 and AVR1-like were further analysed by using Agrobacterium-mediated transient expression assays and infection assays. We also determined the motifs of AVR1 that are required for virulence and for the interaction with the host target Sec5 by transient in planta expression and by yeast two hybrid assays, respectively.

Since host vesicle trafficking related proteins are potential targets of pathogen effectors, **Chapter 6** focuses on the role of other exocyst components in plant defense. Exocyst component genes from *N. benthamiana*, tomato and potato were retrieved, their sequences were aligned and used for phylogenetic analyses. Constructs for virus induced gene silencing were made and gene function analysis in *N. benthamiana* and tomato was performed to investigate the role of each exocyst component in plant defense against *P. infestans* and *P. capsici*.

In **Chapter 7** of this thesis, effector host targets and effector-modulated host defense are discussed in a broader context.

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Chapter 2

Recognition of the *Phytophthora infestans* RXLR effector AVR1 by its matching resistance gene *R1* is determined by specific motifs and regions in the C-terminus

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Abstract

Oomycete RXLR effectors are fast evolving proteins with a rather conserved N-terminal RXLR motif and diverse C-terminal effector motifs. Three motifs can be recognized in the C-termini of RXLR effectors, named W, Y and L motifs. *Phytophthora infestans* RXLR effector AVR1 and its close homologue AVR1-like (A-L) both contain two W motifs and one Y motif. AVR1 triggers a potato R1-mediated hypersensitive response, whereas A-L does not. In this study, we show that both the Y motif and the C-terminal T-region are important to induce R1-mediated recognition. Although the T-region itself is not sufficient to trigger R1 recognition, it enables recognition of A-L. Moreover, a version of AVR1 that lacks the T-region still triggers R1-mediated HR, indicating that AVR1 is likely recognized via two independent regions. These data show that A-L circumvents R1 recognition not only by deletion of the T region, but also by mutation of the conserved C-terminal effector motifs.

Introduction

Plant pathogenic oomycetes cause devastating diseases on a wide range of host plants, including crops, ornamentals and trees, and this results in significant losses in agriculture and damage to natural ecosystems. The most notorious oomycete plant pathogens belong to the genus *Phytophthora*, which includes over 120 species (Kroon et al., 2012). One of the best studied and economically important species is *Phytophthora infestans*, the causal agent of potato late blight and a major yield limiting factor in potato production.

Basal defense against plant pathogens is largely mediated by pattern recognition receptors (PRRs) that are localized at the plasma membrane and function in surveillance of exogenous pathogen-associated molecular patterns (PAMPs) to induce PAMP-triggered immunity (PTI) (Dodds and Rathjen, 2010). In addition, race-specific resistance is mediated by NLR proteins; intracellular immune receptors containing conserved nucleotide-binding and leucine-rich repeat domains (NLR), that are activated through recognition of pathogen effectors to initiate effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI is normally associated with a rapid local cell death known as the hypersensitive response (HR) to halt pathogen colonization. NLRs can be divided into two major subfamilies according to the presence of either a Toll/interleukin-1 receptor-like domain (TIR; Meyers et al., 1999) or a coiled coil domain (CC) at the N-terminus (Pan et al., 2000). Thus far, all identified potato late blight resistance genes encode NLR proteins belonging to the CC-subfamily (Vleeshouwers et al., 2011).

Multiple late blight resistance genes have been identified in wild *Solanum* species and successfully introgressed into different potato cultivars (Black et al., 1953; Malcolms and Black, 1966). The first cloned late blight resistance gene is *R1*, which originates from *Solanum demissum* (Ballvora et al., 2002). Despite the enormous efforts in resistance breeding, *R1*-containing potato cultivars have nowadays little agricultural value. Due to many years of co-evolution, the majority of the current *P. infestans* isolates are virulent on *R1*-containing potato cultivars. This is due to the fact that the avirulence effector AVR1 is no longer produced in most of the isolates. *Avr1*, the corresponding avirulence gene for *R1*, was identified by map-based cloning and encodes an RXLR effector (Guo, 2008; Govers et al., unpublished data). *P. infestans* strains that have the *AVR1* gene are not able to cause disease on *R1*-containing potato plants, whereas strains that are

virulent on *R1* plants lack *AVR1*. These strains, however, contain an *AVR1-like* (*A-L*) gene instead of *AVR1*. A-L is a close homologue of AVR1 and has a premature stop codon resulting into a 38 amino acid truncation at the C-terminus. AVR1 triggers an R1-mediated HR when it is co-agroinfiltrated with R1 into the leaves of *N. benthamiana* plants, however, A-L can not trigger R1-mediated HR.

So far, all cloned *P. infestans* avirulence genes encode RXLR effectors, small proteins secreted during the infection process that modulate host defense. RXLR effectors are solely found in oomycetes and are named after a conserved N-terminal amino acid motif composed of arginine-any amino acid-leucine-arginine (RXLR) (Vleeshouwers et al., 2011). Various *P. infestans* RXLR effectors are found to act as avirulence factors and as such, are recognized specifically by a corresponding potato NLR protein. AVR1 for example, is recognized by R1 and AVR2 by R2 (Vleeshouwers et al., 2011). The *P. infestans* genome contains over 550 RXLR effector genes, of which most are located in gene-sparse and repeat-rich regions, that evolve more rapidly in comparison to genes in gene-dense regions (Haas et al., 2009). Hence, RXLR effector genes are able to undergo rapid adaptation thereby avoiding recognition by resistance proteins. *P. infestans* can overcome *R*-gene mediated resistance by adapting its matching effectors in various ways. For example, by mutation of just two amino acids in AVR3a ($K^{80}I^{103}$ to $E^{80}M^{103}$), *P. infestans* can overcome R3a-mediated recognition (Bos et al., 2006). In the case of AVR4, there is a frame shift mutation in the coding region of the *AVR4* gene, which results into a truncated peptide that is no longer recognized by R4 (van Poppel et al., 2008). *P. infestans* is also able to secrete effectors that suppress ETI, for example the RXLR effector IPI-O4 which suppresses IPI-O1-triggered Rpi-blb1-mediated HR (Champouret et al., 2009; Halterman et al., 2010).

The conserved RXLR motif in the N-terminus of RXLR effectors is required for host translocation of RXLR effectors, whereas the diverse C-termini are mostly required for effector functions (Whisson et al., 2007; Dou et al., 2008). Three conserved motifs, termed W, Y and L motifs, can be identified in the C-terminal regions of RXLR effectors (Jiang et al., 2008). The role of these conserved C-terminal motifs in inducing effector-triggered HR was so far analysed for a number of AVR effectors, including AVR-blb1, AVR-blb2, AVR4 and AVR3a from *P. infestans* and AVR1b from *P. sojae* (Oh et al., 2009; Champouret et al., 2009; van Poppel et al., 2008; van Poppel et al., 2009; Bos et al., 2006; Dou et al.,

2008), and these studies showed that some of the conserved motifs are essential to initiate ETI. For example, *P. infestans* AVR4 contains three W motifs, of which W2 in combination with either W1 or W3 can trigger HR on R4-containing potatoes (van Poppel et al., 2009).

Both AVR1 and A-L contain two W motifs and one Y motif (Govers et al., unpublished data). However, the functions of these motifs are not known. To understand the role of conserved motifs in the C-termini of AVR1 and A-L in triggering R1-mediated resistance. We generated motif deletion and chimeric constructs and tested these by transient expression in *N. benthamiana* for their ability to trigger the R1-mediated HR.

Results

The primary structure of AVR1 and AVR1-like

RXLR effectors are proteins secreted by oomycete pathogens that are typically equipped with a signal peptide at the N-terminus followed by a RXLR motif and a C-terminal region. In previous analysis three conserved motifs including two W motifs namely W1 and W2, followed by a Y motif were identified in the C-termini of AVR1 and its close homologue AVR1-like (A-L) (Govers, unpublished data) (Figure 1). Sequence alignment of AVR1 and A-L is shown in Figure S1. Compared to A-L, AVR1 contains a 38 amino acid extension at the C-terminus, hereafter named Tail-region (T-region). Linker regions between the conserved C-terminal motifs are designated linker 1 (ln1) and linker 2 (ln2), respectively.

To determine the activity of AVR1 we agroinfiltrated *N. benthamiana* leaves to co-express AVR1 and R1 and monitored HR. To optimize the agroinfiltration assay we first tested whether or not the presence of the native signal peptide of AVR1 interferes with R1-mediated recognition. AVR1 with and without its native signal peptide and equipped with a GFP-tag, were co-agroinfiltrated with R1 into *N. benthamiana* leaves. Results show that both AVR1^{+}-GFP (with signal peptide) and AVR1-GFP (without signal peptide) are able to trigger R1-mediated HR. Dilution series were made to compare the different strengths of AVR1^{+}-GFP and AVR1-GFP in triggering R1-mediated HR. This revealed that AVR1-GFP is able to trigger HR at a lower concentration (Figure S2), indicating that the native signal peptide of AVR1 is likely secreting AVR1 into the apoplast and that translocation of AVR1 into the host becomes the limiting factor. At a certain concentration the secreted AVR1 does no longer trigger R1-mediated HR, while at the same concentration the AVR1

that is retained in the cytoplasm is still able to do so. As a result, all the constructs we generated for further analysis were made without the signal peptide.

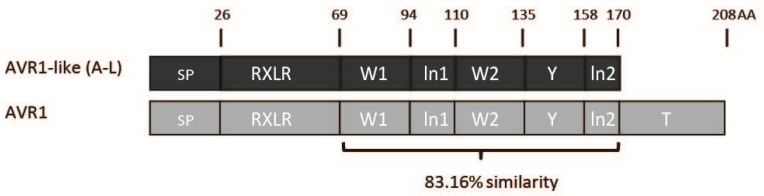

Figure 1. Schematic cartoon showing the primary structure of AVR1 and A-L.
The signal peptide (SP), conserved motifs (RXLR, W1, W2, Y), linker regions (ln1, ln2) and T-region have been indicated with amino acid positons. Sequence similarity was determined using SIAS (http://imed.med.ucm.es/Tools/sias.html).

The T-region of AVR1 is important but not sufficient to trigger R1-mediated HR

In order to investigate which C-terminal motifs of AVR1 are needed to trigger R1-mediated HR various deletion and chimeric constructs were made, which were then co-expressed with R1 in *N. benthamiana* leaves. Since AVR1 contains the 38 amino acid T-region at the C-terminal end, we first tested whether the T-region (T) of AVR1 is required for R1 recognition. A version of AVR1 that lacks the T-region (AVR1-ΔT) was still able to induce R1-mediated HR in 100% of the infiltrated sites, but the HR development was delayed for 1 day when compared to AVR1 (Figure 2). In contrast, the T-region itself was not able to trigger R1-mediated HR (Figure 2), but, when the T-region was fused to A-L the chimeric protein showed HR inducing activity albeit the HR was delayed. Together, these findings indicate that the T-region of AVR1 contributes to R1-mediated HR.

In order to investigate whether other conserved motifs in the C-terminus of AVR1 are responsible for R1-mediated HR, additional motif deletion and chimeric constructs between AVR1 and A-L were made. R1 was transiently co-expressed with these constructs in the leaves of *N. benthamiana*. Results show that deletion of the W1 motif from AVR1 does not influence recognition by R1. However, deletion of either W2, Y or Ln2 totally abolishes R1-mediated recognition (Figure 2). Since deletions may change the protein structure, we exchanged the corresponding W2, Y and both Ln-regions from A-L to AVR1. All these motif chimeric constructs which have the same length as native AVR1 can trigger R1-

mediated HR. This indicates that W1, W2 and Y motifs are more important to maintain the AVR1 structure rather than to trigger R1 recognition independently. To confirm this, we exchanged these motifs either separately or together with the corresponding motifs in A-L. None of these chimeric constructs was able to induce R1-mediated HR in *N. benthamiana* (Figure 2), indicating that these conserved motifs by itself do not contain determinants for triggering R1-mediated HR. We have shown that the effector domain of AVR1 without its T-region is sufficient to trigger R1-mediated HR, and that the T-region enhances this recognition. All together these results suggest that R1 is likely recognizing two epitopes in AVR1, the C-terminal region contains these conserved motifs as one epitope and the T-region as the other. The truncation of the T-region in A-L and the nucleotide polymorphisms between AVR1 and A-L are all essential for avoiding R1-mediated HR.

The T-region of AVR1 trans-complements A-L to trigger R1-mediated HR

The T-region of AVR1 was found unable to trigger R1-mediated HR (Figure 2 and 3A), and this could be caused by its instability *in planta*. To rule out this possibility, an N-terminal GFP tag was fused to the T-region (GFP-T) , which enabled to monitor protein stability by western blot analysis. As shown in Figure 3B GFP-T is stable. This indicates that although GFP-T is stable *in planta* (Figure 3B and 3C), it is still not sufficient to trigger R1-mediated HR upon co-expression with R1 in leaves of *N. benthamiana* (Figure 3A, left panel). However, HR development was found when we co-expressed the T-region with A-L and R1 (Figure 3A, middle and right panel), which indicates the importance of the T-region in R1 recognition. This result also suggests that there is trans-complementation of A-L by the T-region. This could be due to an association between the T and A-L, which as such may mimic the AVR1 structure. To test whether the T-region can bind with A-L *in planta*, co-immunoprecipitation assays were performed. Results show that AVR1 is able to co-immunoprecipitate with GFP-T, whereas A-L is not (Figure 3B).

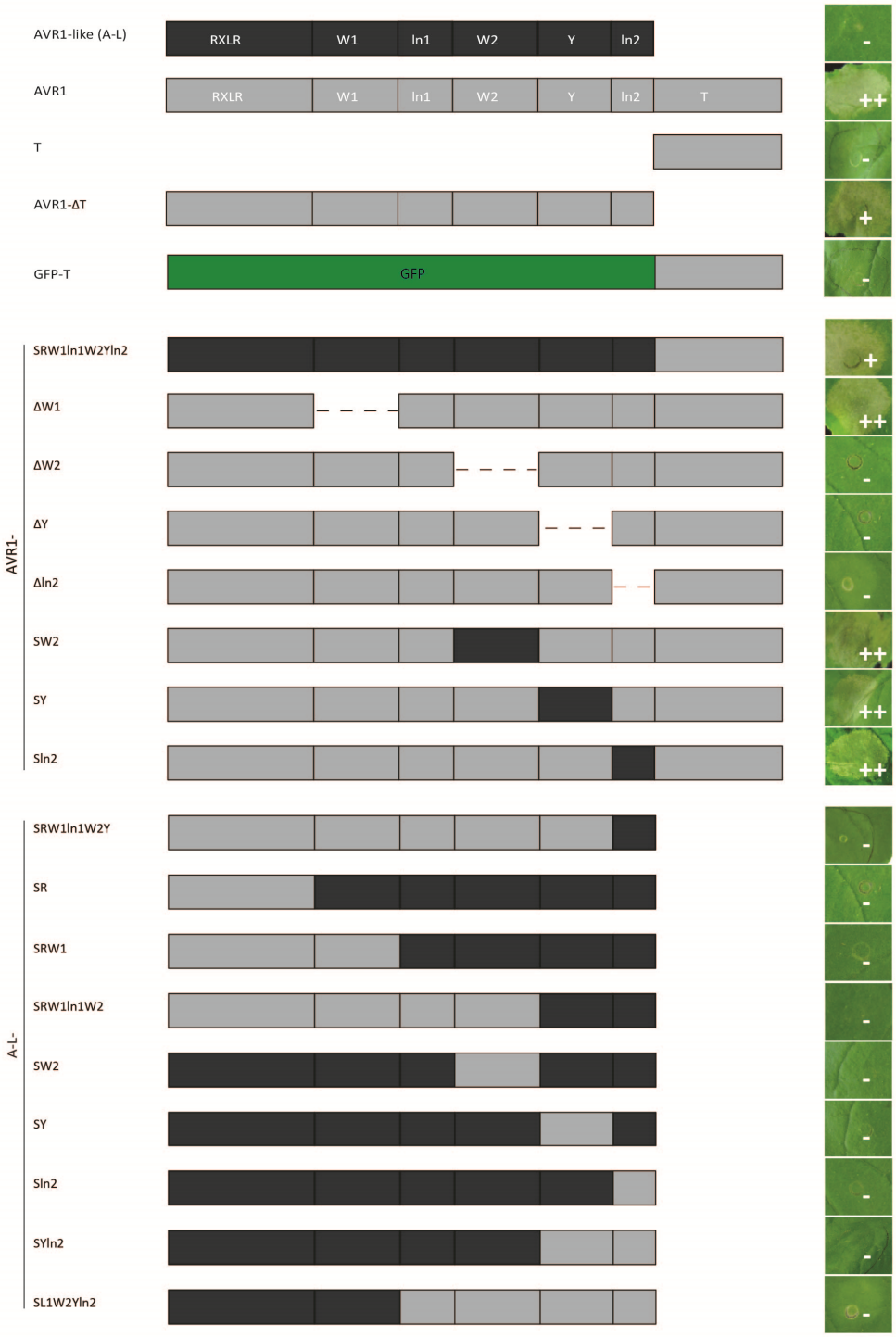

Figure 2. The T-region of AVR1 is important but not sufficient for R1 recognition. *Agrobacterium* stains containing AVR1, A-L and AVR1 truncated and chimeric constructs were co-agroinfiltrated with R1 in *Nicotiana benthamiana* leaves. The final OD of the *Agrobacterium* suspension was adjusted to 0.5. Pictures of the infiltrated leaves at 3 dpi are shown on the right. Experiments were repeated three times with a total of 19 infiltrated sites per construct, and the HR development in this assay is either 0% or 100% for all infiltrated sites. ++: HR at 2 dpi; +: HR at 3 dpi; -: no HR.

Since we repeatedly observed a higher protein accumulation of AVR1 than of A-L, we hypothesized that R1-mediated recognition of AVR1 could be induced by the amount of effector proteins present in the cell. It could be that the T-region stabilizes A-L in such a way that the A-L protein accumulation increased. To test this, we co-expressed GFP-T with AVR1-myc, A-L-myc or IPI-O1-myc. GFP-HA co-expressed with the same three effectors was included as control. Proteins were isolated two days after infiltration and subjected to western blot analysis. Results show no difference between A-L protein levels in the presence of either GFP-T or GFP-HA (Figure 3C), which suggests that the presence of the T-region does not alter A-L protein levels. To investigate the effect of the amount of T-region in triggering HR, we changed the relative ratios in the agroinfiltration mixture in such a way that the relative amount of the T-region would increase. However, increasing the relative concentration of the T-region did not enhance the HR upon agroinfiltration (Figure S3).

Most chimeric A-L constructs can be trans-complemented with the T-region of AVR1

Since all the constructs used in the HR assays do not contain a tag for monitoring the protein stability, it might be possible that those constructs which cannot induce an R1-mediated HR produce unstable proteins rather than lacking functional important motifs. Hence, we co-agroinfiltrated the A-L motif chimeric constructs that are as such not able to trigger R1 recognition, with the T-region. Results show that apart from constructs A-L-SRW1 and A-L-SRW1ln1W2, all constructs could be trans-complemented to induce HR (Figure 4). This indicates that most of these proteins are stable and that their loss of function is due to lack of functional important motifs. For A-L-SRW1 and A-L-SRW1ln1W2, it cannot be excluded that the lack of recognition is due to less stable proteins.

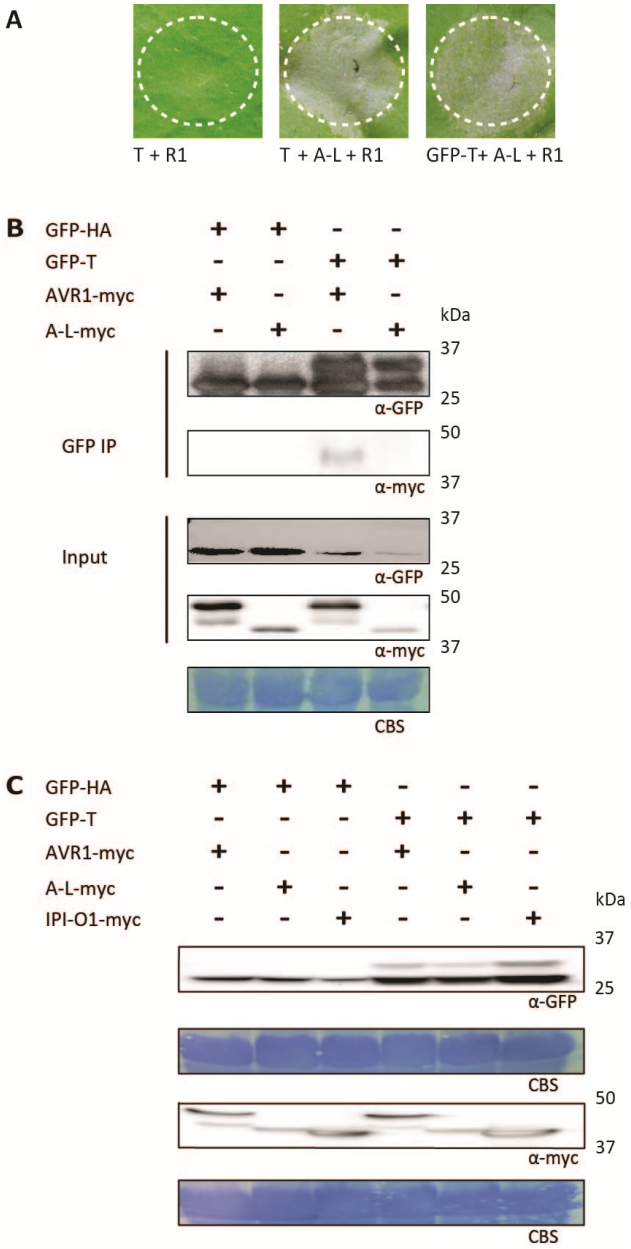

Figure 3. The T-region of AVR1 trans-complements A-L to trigger R1-mediated HR. (A) HR development upon co-agroinfiltration of different combinations of R1, A-L, GFP-T and T in *N. benthamiana* leaves. Pictures were taken at 3 dpi. Dotted lines indicate agroinfiltrated zones. (B) GFP-T, GFP-HA, AVR1-myc and A-L-myc were co-agroinfiltrated in *N. benthamiana* leaves in different combinations. GFP-tagged proteins were immunoprecipitated using GFP-trap_A® beads (IP: GFP). GFP- and myc-tagged proteins were detected using α-GFP or α-myc antibodies. IP, Immunoprecipitation; CBS, Coomassie blue staining visualizing the 50 kDa RuBisCO band indicating equal loading. (C) Immunoblot showing accumulation of myc-tagged AVR1, A-L and IPI-O1 when co-expressed with either GFP-T or the control GFP-HA. Total proteins were isolated at 2 dpi and tagged proteins were detected by immunoblotting using either α-GFP or α-myc antibodies.

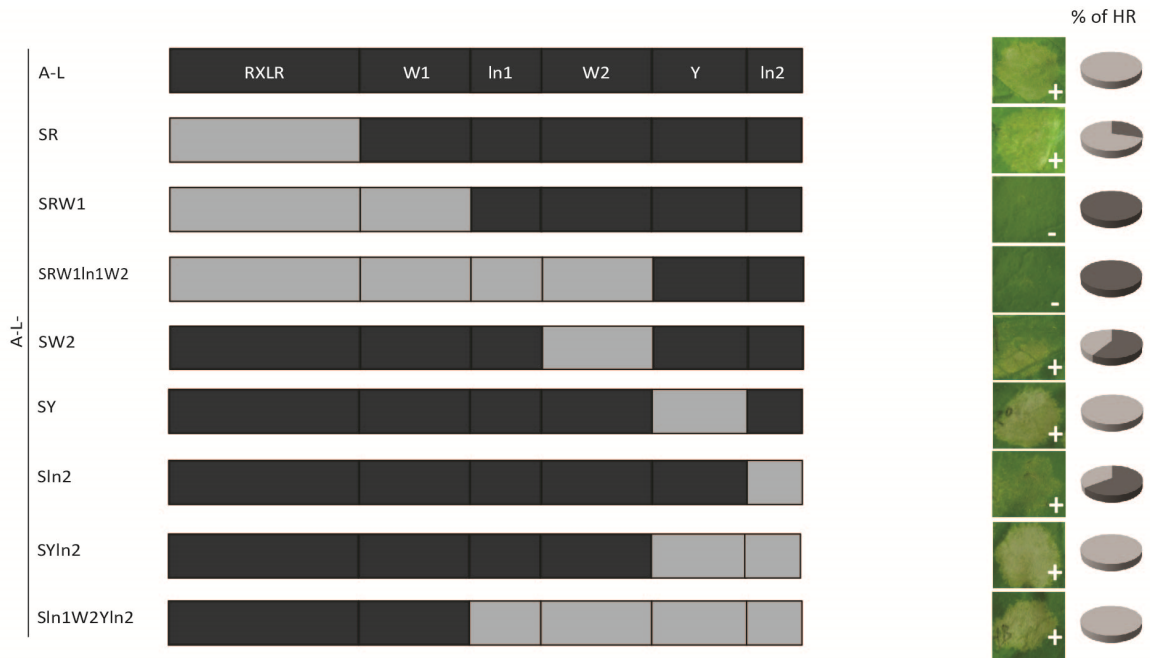

Figure 4. The T-region of AVR1 trans-complements A-L chimeric constructs.
AVR1 mutants were co-agroinfiltrated with R1 and T-region into *N. benthamiana* leaves. At 3 dpi, HR was scored and pictures were taken. Experiments were repeated three times with a total of 15 infiltrated sites per construct. Pie charts show the percentages of HR response in which light grey indicates HR and dark grey no HR, respectively.

Discussion

Several studies on RXLR effectors have shown that their C-terminal half determines effector functionalities, specifically in promoting pathogen colonization and triggering corresponding R protein-mediated recognition. For example, the 75-amino acid C-terminal half of *P. infestans* AVR3a is responsible for R3a-mediated HR, and shown to be essential for the suppression of INF1-induced cell death in *N. benthamiana* (Bos et al., 2006). For *P. infestans* AVRblb2 a 34 amino acid long region in its C-terminal half is sufficient to trigger Rpi-blb2-induced HR (Oh et al., 2009). The W and Y motifs found in the *P. sojae* effector AVR1b were reported to be important for the induction of RPS1b-mediated HR (Dou et al., 2008). In this study, we analysed which motifs of AVR1 are required for R1 activation. By motif deletion, we determined that the W1 motif in AVR1 is not essential for triggering R1 recognition, as deletion was not altering HR development. In contrast, deletion of the C-terminal motifs W2 and Y abolished R1-mediated HR as did In2, and this indicates that these motifs or linker regions

in AVR1 are essential for R1 recognition. However, when these motifs and linker regions were exchanged with corresponding motifs from A-L, the chimeric proteins were still triggering R1-mediated HR as efficiently as unaltered AVR1. Moreover, when fusing the T-region to A-L, the chimeric protein was also able to trigger R1-mediated HR. As a result we think that none of these three W2, Y and ln2 domains by itself determine recognition by R1, but instead function together to trigger R1-mediated HR. The T-region, which has no sequence homology to any other RXLR effector, also appeared to be important for activation of R1-mediated recognition. Although deletion of the T-region did not impair AVR1ΔT activity, the development of HR is delayed. However replacement of any motif or linker regions in the AVR1ΔT construct with the corresponding motifs or regions from A-L abolished the ability to trigger R1-mediatd HR. This indicates that all these conserved motifs and linker regions are needed for triggering R1-mediated HR, and that it is not possible to pinpoint one specific region as the determinant for R1 recognition. Although the T-region by itself is not sufficient to trigger R1 recognition, it has a prominent role in recognition, as is also shown by the observation that the T-region can modify the activity of A-L; either fused as a tail to A-L or coinfiltrated with A-L, it causes an R1 mediated HR. Apparently R1 recognizes two epitopes in AVR1, one in the C-terminal regions that contains these conserved motifs and the linker regions, and one in the T region.

To understand how this recognition of A-L works, we checked whether the T-region associates with A-L to complement it. Results show that this complementation is not due to direct interaction of the T-region with A-L, as co-immunoprecipitation experiments fail to detect an interaction between the T-region with A-L. Although in infiltration experiments A-L protein levels are lower than AVR1 protein levels, we could show that R1-mediated HR triggered by A-L together with the T-region is not due to increased protein levels of A-L.

Taken together, it appears that R1 recognizes at least two epitopes in AVR1; one in the C-terminal conserved motifs and one in the T-region. Compared with AVR3a, for which *P. infestans* has point mutations to circumvent R3a recognition (Bos et al., 2006), our results indicate that for AVR1 point mutations are not sufficient to overcome R1-mediated resistance. To circumvent recognition *P. infestans* deleted AVR1. The evidence for this is that from the *P. infestans* isolates we have analysed so far, the ones which are able to colonize R1-containing potato

all lost the *AVR1* gene in their genome, but instead have gained A-L at a different locus (unpublished data).

Material and methods

Plasmid construction

Effector genes *AVR1* (PITG_16663.2) and *A-L* (*AVR1-like*; PITG_06432.1) were PCR amplified using appropriate primers (Supplemental Table 1) from genomic DNA of the *P. infestans* isolates 88029 and 88133, respectively. PCR fragments were cloned into the Gateway vector pENTR/D-TOPO (Invitrogen), and recombined into the plant binary vectors pGWB20 (Nakagawa et al., 2007) and pSOL2095 (Liebrand et al., 2012) using Gateway LR Clonase II (Invitrogen). Resulting plasmids were named AVR1-myc, A-L-myc, AVR-GFP and A-L-GFP, respectively. PCR amplicons encoding the T-region of AVR1 were amplified from plasmid AVR1-myc and cloned into pSOL2094, resulting into plasmid GFP-T. Motif deletion and chimeric constructs were constructed by overlap extension PCR using appropriate primers (Supplemental Table 1), and subsequently cloned into pGRAB using *Not*I and *Asc*I restriction sites. Binary plasmids were transformed via electroporation into *Agrobacterium tumefaciens* strain AGL1.

Plant growth conditions and agroinfiltration

N. benthamiana plants were grown in the greenhouse under standardized conditions. Middle leaves from 4-to-5 week old plants were used for agroinfiltration. Transgenic *A. tumefaciens* strains were grown in YEB medium (per liter: 5 g beef extract, 1 g yeast extract, 5 g peptone, 0.5 g $MgCl_2$) at 28°C with appropriate antibiotics for 18 to 24 hours. Agrobacteria were resuspended in infiltration medium (per liter: 10 mM MES pH 5.6, 5 g MS salts (w/o vitamins), 20 g sucrose, and 150 µM acetosyringone) and adjusted to a final OD600 of 1 prior to agroinfiltration of *N. benthamiana* leaves. For HR assays, corresponding AGL1 strains were mixed in a 1:1 or 1:1:1 ratio.

Protein isolation and co-immunoprecipitation

Proteins were isolated from *N. benthamiana* leaves 2 days after agroinfiltration. Leaf material was grinded in liquid nitrogen and one gram of grinded sample was incubated with 2 ml of extraction buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 5 mM DTT, and 1 proteinase inhibitor tablet (Roche) per 50 mL) for 30 min

on ice. Cell debris was pelleted and 100 µl supernatant was kept as input sample. Remaining volumes were incubated with 15 µl GFP-trap_A® (ChromoTek) beads at 4°C for 2 hours. Subsequently, the beads were collected by centrifugation at 720g for 2 min and washed for 8 times with extraction buffer. After washing, the beads were collected and pipetted into a new 1.5 ml tube containing 100 µl extraction buffer. Protein samples were boiled in 4x loading buffer (200 mM Tris-HCl pH 6.8, 8% SDS, 400 mM DTT, 40% glycerol and 0.2% bromophenol blue) for 5 min before gel electrophoresis.

Immunoblotting and protein detection

Proteins separated by SDS-PAGE were transferred to Immune-Blot® PVDF membrane (Bio-Rad), and subsequently incubated with a blocking buffer containing 5% skimmed milk powder at room temperature for two hours. Membranes were incubated with α-GFP-HRP (Miltenyi Biotec, 130-091-833) or α-myc (Sigma-Aldrich, 9E10) antibodies for approximately two hours. Myc-tagged proteins were hereafter probed with HRP-conjugated anti-mouse IgG secondary antibody (Amersham). Protein bands were visualized with SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific). Membranes were stained with coomassie blue to visualize RuBisCO proteins.

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Supplementary information

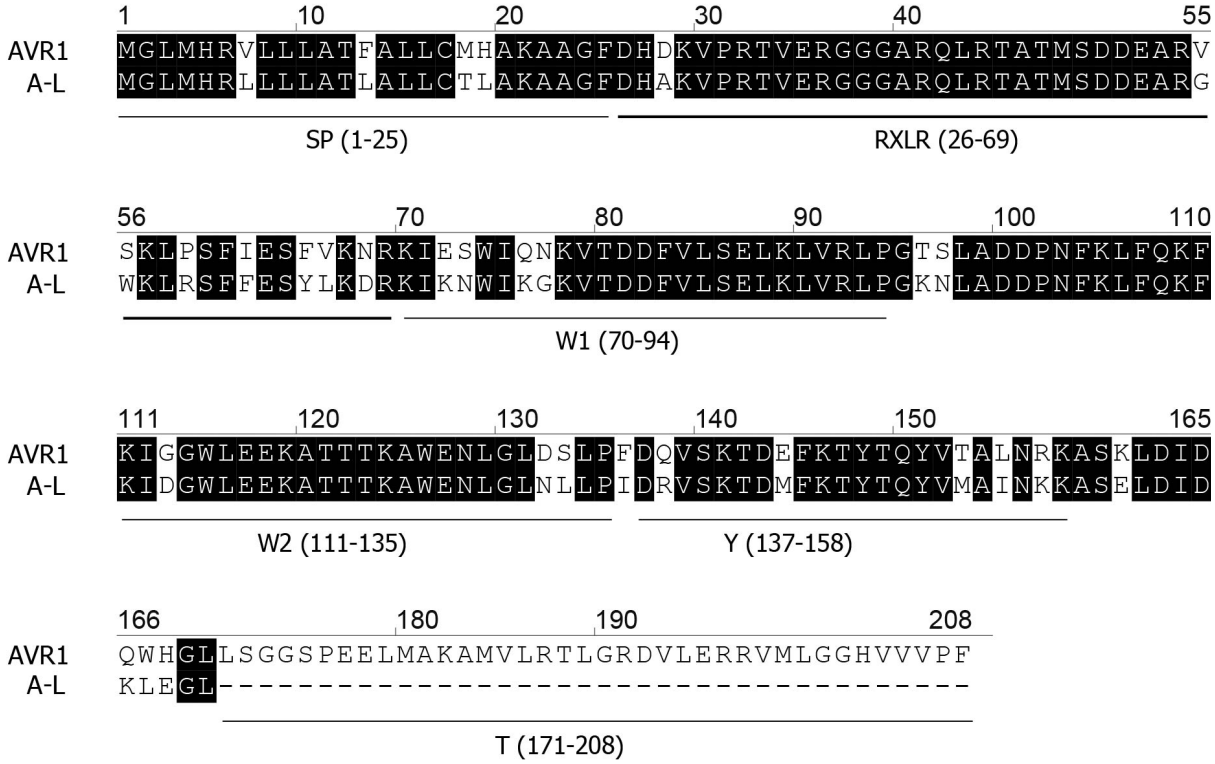

Figure S1. Sequence alignment of AVR1 and AVR1-like (A-L).

The protein sequences of AVR1 and A-L were aligned with Vector NTI. The signal peptide (SP), conserved motifs, linker regions and T-region are indicated below the alignment.

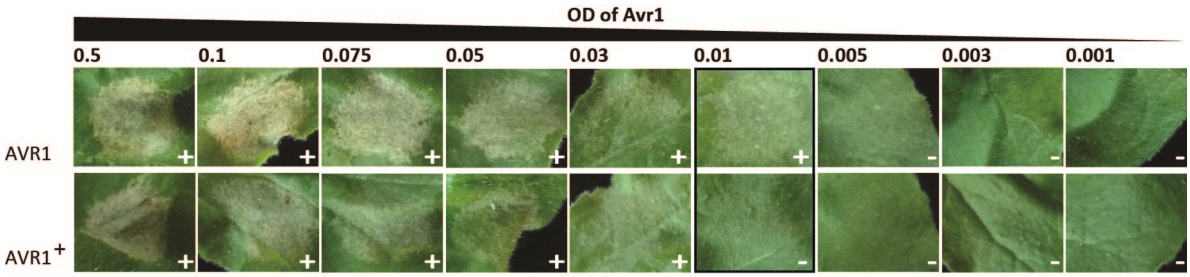

Figure S2. AVR1 does not require its signal peptide to trigger R1-mediated HR.
Both AVR1^{+}-GFP and AVR1-GFP (without native signal peptide) induce HR upon co-expression with R1 in *N. benthamiana*. Concentrations of AVR1 were adjusted to different final ODs as indicated, and R1 was consistently kept at the final OD of 0.5. Experiments were repeated three times with in total 15 infiltrated sites per construct. Pictures were taken at 3 dpi.



Figure S3. R1-mediated HR is not enhanced by increasing the concentration of T-region.
N. benthamiana leaves were co-agroinfiltrated with A-L, T, and R1 in different volume ratios as indicated. Pictures were taken at 3 dpi.

Table S1. Primers used in this study.

Primer name	Sequence (5'-3')
AVR1-R	gtgcggccgcttaaaatggtaccacaacatgtccacc
AVR1ΔSP-F	gaggcgcgccatggaccacgacaaggttccaag
A-L-R	aaatggtaccacaacatgtccacc
A-LΔSP-F	caccatggccggattcgaccac
A-LSR-R	ccaattcttgatttt tcggtttttaacgaaggactcg
A-LSR-F	cgttaaaaaccgaaaaatcaagaattggatcaagggc
AVR1SRW1-R	ggttcttcccgggcaatctcacgagttttagc
AVR1SRW1-F	gagattgcccgggaagaacctggcggacg
A-LSIn1W2YIn2-R	ggctcgttccaggcaatctcacgagttttagc
A-LSIn1W2YIn2-F	gagattgcct ggaacgagcctggcggacg
A-LSRW1In1W2-R	gctcactcgatcaattgggagtgaatccaagcc
A-LSRW1In1W2-F	ggattcactcccaattgatcgagtgagc
A-LSYIn2T-R	gctcacttgatcaaatgggagtaaattcaagcc
A-LSYIn2T-F	gcttgaatttactcccatttgatcaagtgagc
AVR1ΔT-R	cctccacttcacagcccgtgcc
A-LST-R	ggtgatcctccacttaacagcccctcc
A-LST-F	gttggaggggctgttaagtggaggatcacc
A-LSIn2T-R	cgagtttgcttgccttcttgttaatcgc
A-LSIn2T-F	gcgattaacaagaaggcaagcaaactcg
AVR1SW2-R	gccgtcaatcttaaacttttgaaagagc
AVR1SW2-F	gctctttcaaaagtttaagattgacggctgg
A-LSW2-R	ccgccaatcttaaacttttgaaagagc
A-LSW2-F	gctctttcaaaagtttaagattggcggctgg
A-LSY-R	gcttgccttcctgttaagcgc
A-LSY-F	gcgcttaacaggaaggcaagcgaactcg
A-LSRW1In1W2In2-R	taacagcccgtgcaacttgtc
T-F	cggaattcatgttaagtggaggatcacctgag
T-R	gtgcggccgcttaaaatggtaccacaacatgtcc
T-gatewayF	caccatgttaagtggaggatcacctgag
T-gatewarR	ttaaaatggtaccacaacatgtcc

Chapter 3

Immune activation mediated by the late blight resistance protein R1 requires nuclear localization of R1 and AVR1

Yu Du, Klaas Bouwmeester, Jeroen Berg, Francine Govers

Abstract

Phytophthora infestans is an oomycete plant pathogen causing late blight on potato. Late blight resistance largely relies on intracellular nucleotide-binding leucine-rich repeat (NLR) proteins that recognize matching avirulence (AVR) proteins from the pathogen. All known *P. infestans* AVR proteins are intracellular RXLR effectors. The mechanisms underlying NLR-mediated late blight resistance are poorly understood. In this study we focussed on the *P. infestans* RXLR effector AVR1 and its matching potato NLR R1 and addressed the question in which subcellular compartment effector perception and defense activation takes place. Analyses of the subcellular localization of AVR1 and R1 *in planta* revealed that they are not restricted to one compartment; both were found in the nucleus and in the cytoplasm. By using peptide-based nuclear localization and export signals (NLS/NES) we artificially modified the nucleocytoplasmic partitioning of R1 and AVR1 and studied the effect on R1-AVR1 recognition. This revealed that activation of R1-mediated immunity only takes place when both, AVR1 and R1 are localized in the nucleus. In the absence of matching NLRs, RXLR effectors often act as virulence factors e.g. by supressing host defense. By perturbing the nucleocytoplasmic partitioning of AVR1 we could show that AVR1-mediated suppression of cell death is dependent on localization of AVR1 in the host cytoplasm.

Introduction

During evolution plants have developed two classes of immune receptors for defense against a wide range of pathogens. The first class consists of pattern recognition receptors (PRRs), which are localized at the plant plasma membrane and can detect so-called pathogen-associated molecular patterns (PAMPs). This detection leads to PAMP-triggered immunity (PTI) that inhibits pathogen colonization (Chisholm et al., 2006). The second class of immune receptors comprises the intracellular resistance (R) proteins, which detect pathogen-derived effectors and mediate effector-triggered immunity (ETI) (Hardham and Cahill, 2010). ETI is often associated with a strong hypersensitive response (HR) at the host infection site to hamper pathogen colonization. Most of these intracellular R proteins share a central nucleotide-binding (NB) domain and a C-terminal leucine-rich repeat (LRR) domain and are known as NLR proteins. NLR proteins can be divided in two subclasses according to their N-terminal domain composition (Tameling and Takken, 2008; Belkhadir et al., 2004; Chang et al., 2013). One subclass has a Toll/Interleukin1 Receptor (TIR) domain preceding the NB domain (T-NLR), while the other has a coiled-coil (CC) domain (C-NLR). These two subclasses likely use different protein complexes for downstream immune signalling (Aarts et al., 1998; Century et al., 1997; Feys et al., 2005; Wiermer et al., 2005).

Plant NLRs have two states, the "OFF" and "ON" state (Takken and Tameling, 2009). When NLRs are in the "OFF" state, they are auto-repressed and folded by a conserved chaperone complex that contains HSP90 and its co-chaperones RAR1 and SGT1 (Shen and Schulze-Lefert, 2007; Shirasu, 2009). Upon effector perception, NLR proteins switch to the "ON" state and this triggers nuclear-associated immune responses such as shuttling of proteins over the nuclear membrane, exporting mRNA from the nucleus, and activation or repression of transcription (Shen and Schulze-Lefert, 2007; Shen et al., 2007; Elmore et al., 2011; Chang et al., 2013). This suggests that NLRs are mainly active in the nucleus and likely nuclear localized. This is in line with the observation that 72% of the Arabidopsis NLRs contain a nuclear localization signal (NLS) and are thus predicted to localize in the nucleus (Meyers et al., 2003). Even several NLRs lacking a predicted NLS sequence were found to be nuclear localized, e.g. barley MLA10 and potato Rx (Shen et al., 2007; Tameling et al., 2010).

Not all NLRs however, are restricted to the nucleus; some of them are also found in the cytoplasm (Elmore et al., 2011). To determine if a specific subcellular localization of NLR proteins is required for activation of immune signalling, one can perturb the nucleocytoplasmic partitioning of NLRs by artificial modification with either a NLS that targets the protein to the nucleus or a nuclear export signal (NES) that excludes the protein from the nucleus (Matsushita et al., 2003; Wen et al., 1995). In this way it was shown that immune responses mediated by the barley powdery mildew R protein MLA10 and the tobacco mosaic virus R protein N, are only activated when these NLRs are localized in the nucleus, the site where they interact with transcription factors to regulate defense gene expression (Shen et al., 2007; Burch-Smith et al., 2007; Caplan et al., 2008). Also the Arabidopsis NLR proteins RPS4 and SNC1 have to be in the nucleus to trigger immune responses (Wirthmueller et al., 2007; Cheng et al., 2009). However, some NLRs are activated outside the nucleus. For example for potato Rx, a NLR that confers resistance against potato virus X (PVX), a tightly regulated nucleocytoplasmic partitioning is essential for Rx-mediated immunity. This partitioning is mediated by RanGAP2 that acts as a cytoplasmic retention factor likely via physical interaction with Rx (Tameling and Baulcombe, 2007; Slootweg et al., 2010; Tameling et al., 2010). Arabidopsis RPM1, a NLR protein conferring resistance to the bacterium *Pseudomonas syringae*, activates immunity at the plasma membrane (Gao et al., 2011).

Phytophthora infestans is a filamentous oomycete plant pathogen that causes the devastating late blight disease of potato and tomato. Late blight resistance is governed by NLR proteins. For obtaining late blight resistant cultivars potato breeders exploit NLR genes from wild *Solanum* species and they increasingly make use of NLR-linked DNA markers to facilitate selection (Carrasco et al., 2009; Vleeshouwers et al., 2011). More recently, also transgenic and cisgenic approaches based on cloned NLR genes are applied (Jones et al., 2014; Jo et al., 2014). The first late blight *R* gene that was cloned was *R1* that originates from *Solanum demissum.* It encodes a C-NLR that specifically recognizes *P. infestans* isolates carrying the avirulence gene *AVR1* (Ballvora et al., 2002; van der Lee et al., 2004). All cloned avirulence (*AVR)* genes from oomycete pathogens that match with NLR-type *R* genes in a 'gene-for-gene' manner, encode so-called RXLR effectors. These effectors share the four amino acid motif RXLR in the N-terminus that is characteristic for effectors whose final destination is inside host

cells where they are thought to manipulate host defense responses. Analyses of the subcellular localization of several RXLR effectors showed that they can be found in various subcellular compartments (Caillaud et al., 2012a). Each effector seems to have its own specific destination and presumably in close vicinity of its host target that needs to be modified or inactivated to suppress host immunity (Caillaud et al., 2012b).

Presumably plant NLR proteins must also localize to particular subcellular compartments in order to perceive their matching pathogen effectors. A recent study on *P. infestans* resistance protein R3a showed that both R3a and its corresponding RXLR effector AVR3a localize to the host cytoplasm when either one of the two is present. However, when R3a and AVR3a are both present inside the same cell, they relocalize to endosomal compartments. This relocalization is required for immunity and only takes place in the presence of the KI variant, but not the EM variant of AVR3a. The latter has a virulence function but is not recognized by R3a as avirulence effector (Engelhardt et al., 2012). In another study it was found that the *P. infestans* RXLR effector AVR2 co-localizes with its host target BSL1, a putative phosphatase, and accumulates around the haustorium upon infection (Saunders et al., 2012). AVR2 promotes the association of BSL1 with R2 thereby triggering HR mediated by R2. However, these authors did not address the subcellular localization of R2. Does R2 also co-localize with AVR2 around the haustorium and what is the site in the host cell where the ternary complex is formed? These questions remain to be answered, not only for this R-AVR pair but more in general: where does a NLR protein perceive its matching effector and is co-localization of NLR protein and effector in the same subcellular compartment required to mediate immunity?

In this study, we determined the subcellular localization of the *P. infestans* RXLR effector AVR1 and its matching NLR receptor R1. Both appeared to be present in the nucleus as well as the cytoplasm. For perturbing their nucleocytoplasmic partitioning constructs were made encoding R1 or AVR1 fused to NLS and NES signals and these were transiently expressed in *Nicotiana benthamiana.* Subsequently the effects of this artificial targeting on R1-AVR1 recognition were studied by co-expressing the modified *AVR1* and *R1* genes. This revealed the importance of a proper subcellular localization of both AVR1 and R1 for triggering R1-mediated resistance. By expressing the modified *AVR1* genes in

the absence of *R1*, we could also determine in which subcellular compartment AVR1 is active as an effector with a role in virulence.

Results

R1 localizes in the nucleus and the cytoplasm

In order to determine the subcellular localization of R1, we agroinfiltrated *N. benthamiana* leaves with strain *R1* carrying a binary vector encoding the full length R1 protein with a GFP-tag fused to its N-terminus (construct R1 in Figure S1). The strain *mCherry* that contains a binary vector encoding the free monomeric RFP derivative mCherry was co-agroinfiltrated with *R1* and used as marker to delineate the nucleus and cytoplasm (Lee et al., 2008). Confocal microscopy showed that R1 co-localizes with mCherry indicating that R1 is present in both the nucleus and the cytoplasm (Figure 1A).

To check whether the GFP-tagged R1 (Figure S1) is still functional as R protein, *R1* was co-infiltrated with strain *AVR1* that has a binary vector with the *AVR1* gene (Figure S1). At 3 days post infiltration (dpi) all infiltrated sites showed a Hypersensitive Response (HR) while co-infiltration with a control strain *GFP* never resulted in an HR. This shows that the GFP tag does not prevent R1 to mediate AVR1-triggered HR (Figure 1B). Since HR is not in all cases correlated to resistance (Heidrich et al., 2011), we performed infection assays to investigate whether the GFP-tagged R1 is still able to confer resistance. *N. benthamiana* leaves expressing R1 or GUS were inoculated with *P. infestans* isolate 14-3-GFP which has the *AVR1* gene. Control leaves expressing GUS were successfully infected while leaves expressing R1 showed no lesions (Figure 1C). Apparently, the presence of R1 arrested the growth of *P. infestans* isolate 14-3-GFP demonstrating that transiently expressed R1 is able to mediate resistance and that the GFP tag does not interfere with the function of R1 as R protein.

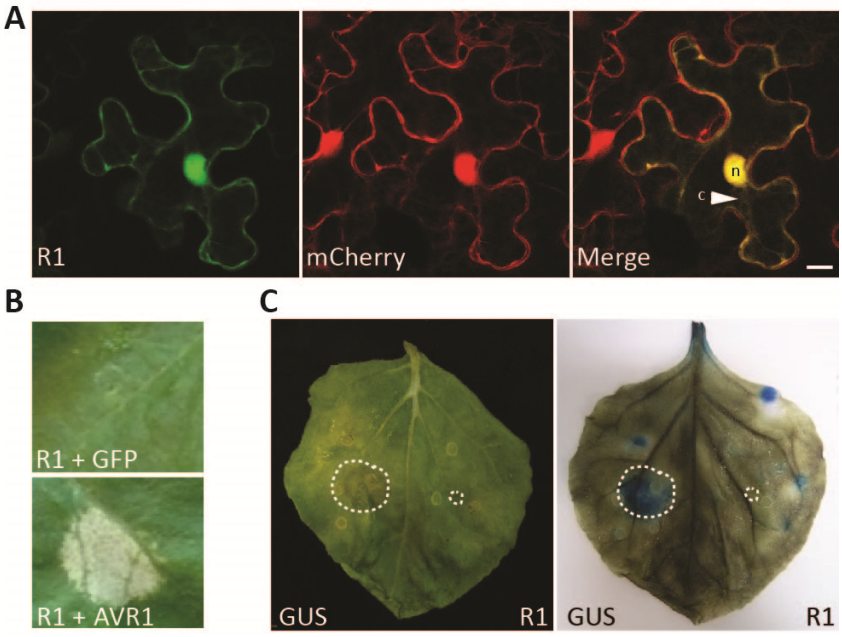

Figure 1. Transient expression of GFP-tagged R1 in *N. benthamiana* leaves.
(A) R1 localizes in the nucleus and the cytoplasm. Confocal microscopy images showing the subcellular localization of R1. *R1* was co-infiltrated with a *mCherry* in a 1:1 ratio and a final OD of 0.1. Pictures taken at 2 dpi show cells co-transformed with R1 (green channel, left panel) and mCherry (red channel, middle panel). In the right panel the two pictures are merged. Bar: 10 µm; c: cytoplasm; n: nucleus. (B) R1 mediates HR upon co-expression with AVR1. *R1* was co-infiltrated with *AVR1* or *GFP* in a 1:1 ratio and a final OD of 0.5. Pictures were taken at 3 dpi. (C) R1 confers resistance towards *P. infestans* isolate 14-3-GFP. *GFP* and *R1* with an OD of 0.3 were agroinfiltrated in the left and right half of the leaf, respectively. At 1 dpi each half of the leaf was inoculated with *P. infestans* isolate 14-3-GFP. Lesion sizes were measured at 6 dai and pictures were taken of lesions before and after trypan blue staining.

R1-mediated HR requires nuclear localization

To investigate whether nuclear localization is needed for R1-mediated immune responses, we artificially targeted the GFP-tagged R1 to the nucleus or the cytoplasm by fusing either a nuclear localization signal (NLS) (Haasen et al., 1999) or a nuclear export signal (NES) (Wen et al., 1995) to R1 ($R1^{NLS}$ and $R1^{NES}$, respectively). Constructs with mutated NLS ($R1^{nls}$) and NES ($R1^{nes}$) fused to R1 were included as controls. Visualization of the GFP-tagged R1 proteins by confocal microscopy showed that $R1^{NLS}$ is indeed efficiently targeted to the nucleus while $R1^{NES}$ is largely exported to the cytoplasm (Figure 2A). As expected $R1^{nls}$ and $R1^{nes}$ behave similar as R1 and are localized in both the nucleus and cytoplasm (Figure

2A). To investigate which subcellular localization is required for R1-mediated HR, we co-infiltrated the various *R1* strains with *AVR1* in *N. benthamiana* leaves, and monitored cell death 3 days later. Of the four R1-AVR1 combinations only the one with $R1^{NES}$ showed no HR (Figure 2B). Since this is also the only one that is restricted to the cytoplasm (Figure 2A) we can conclude that R1 has to be localized in the nucleus to induce AVR1-triggered HR.

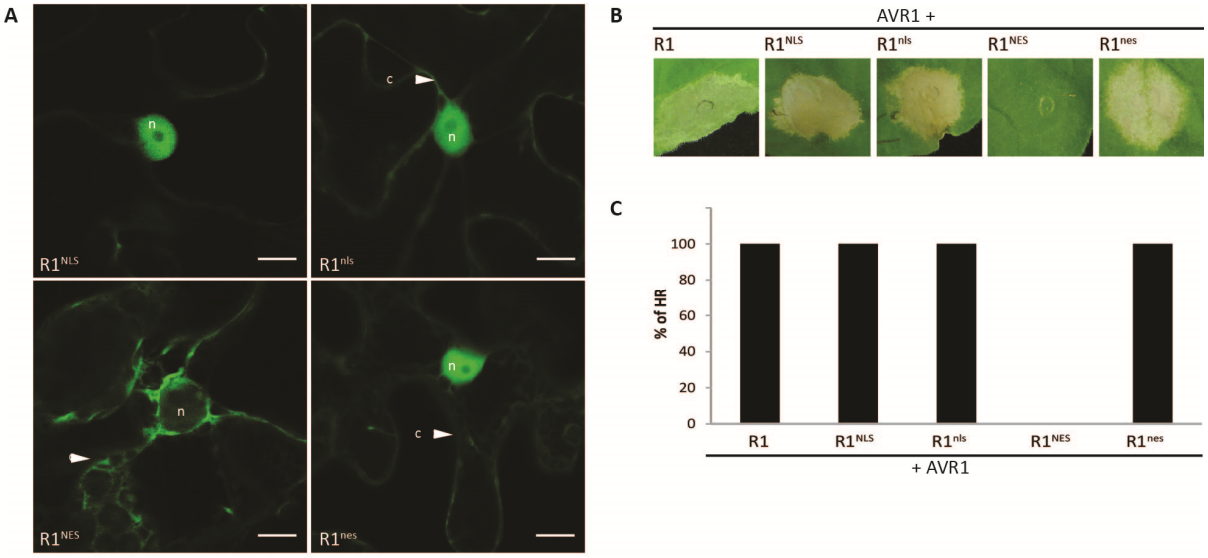

Figure 2. Transient expression of NLS- and NES-tagged R1 in *N. benthamiana* leaves shows that nuclear localization of R1 is required for AVR1-triggered HR.
(A) Confocal microscopy images showing the subcellular localization of R1 modified with the targeting signals NES and NLS and their mutant forms nes and nls. The fusion constructs were agroinfiltrated with an OD of 0.1. Pictures were taken at 2 dpi. Bars: 10 µm; c: cytoplasm; n: nucleus. (B and C) Cytoplasmic targeted R1 loses the ability to mediate HR when co-expressed with AVR1. The strains with the NLS- and NES-tagged R1 constructs were co-infiltrated with *AVR1* in a 1:1 ratio and a final OD of 0.5. Pictures in (B) were taken at 3 dpi. The graph in (C) gives the number of infiltrated sites that showed HR at 3 dpi, out of a total of at least 26 per sample in two independent experiments, and expressed in percentages.

To investigate if nuclear localization of R1 is also required for R1-mediated resistance, we performed infection assays on *R1* agroinfiltrated leaves. One day after agroinfiltration of *N. benthamiana* leaves with a control *GFP* strain, *R1* and the four strains carrying the modified R1 constructs, the leaves were inoculated with zoospores from *P. infestans* isolate 14-3-GFP. Leaves expressing GFP showed expanding lesions at 6 days after inoculation (dai). As expected, leaves expressing R1 showed resistance to this strain (Figure 3A). Lesions were hardly visible and

this was similar on leaves expressing $R1^{NLS}$, $R1^{nls}$ and $R1^{nes}$, the R1 modified versions that are exclusively targeted to the nucleus or show a nucleocytoplasmic distribution similar to R1. In contrast leaves expressing $R1^{NES}$ that targets R1 primarily to the cytoplasm, developed expending lesions similar to the control leaves infiltrated with the GFP-construct (Figure 3A). Quantification showed that about 70% of the GFP-expressing leaves and 50% of the $R1^{NES}$-expressing leaves developed lesions, compared to less than 11% on leaves with R1 localized in the nucleus (Figure 3B). These results show that R1 has to be localized in the nucleus to confer resistance. To determine if the resistance mediated by nuclear localized R1 is specific for AVR1 containing isolates, we also performed infection assays with a *P. infestans* isolate that lacks AVR1. In this assay all the infiltrated leaves developed expanding lesions irrespective of the site where R1 is localized (Figure S2). Due to initial difficulties in cloning, $R1^{NES}$ differs from $R1^{NLS}$, $R1^{nls}$ and $R1^{nes}$ in having its targeting signal at the N-terminus instead of the C-terminus. Later, when the cloning succeeded we were able to show that irrespective of a NES N-terminal ($R1^{NES}$) or C-terminal fusion ($R1^{NES*}$) the results were the same (Figure S3).

Taken together, these results show that nuclear localization of R1 is not only required for initiating an HR triggered by AVR1, but also for arresting growth of *P. infestans* isolates that contain AVR1. This points to a functional connection between R1-mediated HR and R1-mediated resistance.

AVR1 localizes in the nucleus and the cytoplasm

To investigate the subcellular localization of AVR1, we constructed a binary vector with a gene encoding AVR1 (without signal peptide) with a GFP-tag fused to the N-terminus (Figure S1) and co-expressed strain *AVR1* with the free *mCherry* strain in *N. benthamiana* leaves. Confocal microscopy showed that AVR1 co-localizes with mCherry indicating that AVR1 is present in both the nucleus and the cytoplasm (Figure 4A). In a similar way we analysed the subcellular localization of AVR1-like, a close homolog of AVR1 that is not recognized by R1, and found that also this RXLR effector localizes to both nucleus and cytoplasm albeit that the signal in the nucleus is less intense than for AVR1 (Figure 4A). To test if the fluorescence originates from full length GFP-tagged AVR1 and AVR1-like proteins, we isolated proteins from the infiltrated leaves and analysed these on an immunoblot. Probing the blot with GFP-antibodies revealed proteins of the

expected molecular weight of 45 and 41 kDa, respectively (Figure 4B). This shows that both effector proteins are stable *in planta*. To determine if the GFP-tagged AVR1 is still functional as AVR protein, we co-infiltrated *AVR1* with *R1* in *N. benthamiana* leaves and monitored HR at 3 dpi. In contrast to AVR1-like, GFP-tagged AVR1 is able to trigger R1-mediated HR (Figure 4C) and this demonstrates that the GFP-tag does not alter AVR1 activity.

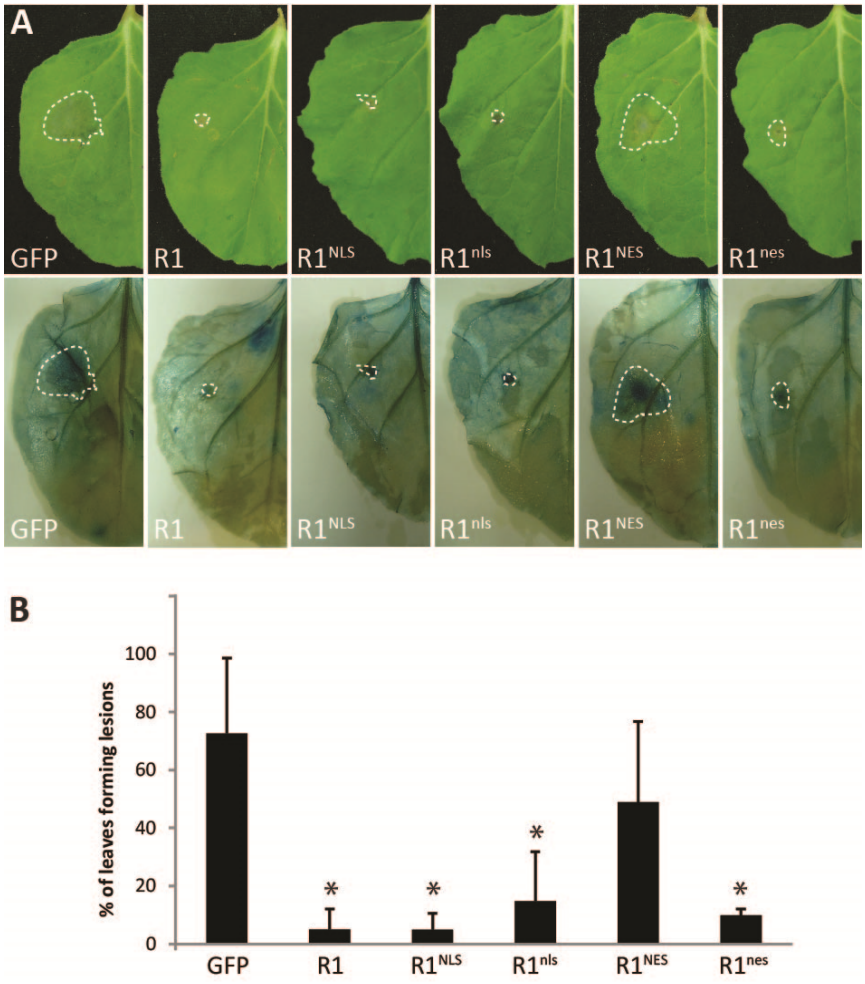

Figure 3. Transient expression of NLS- and NES-tagged R1 in *N. benthamiana* leaves shows that nuclear localization of R1 is required for R1-mediated resistance.
Strains with NLS- and NES-tagged R1 constructs and *GFP* and *R1* as negative and positive control, respectively, were agroinfiltrated with *P19* in a 1:1 ratio and a final OD of 0.3. At 1 dpi, the leaves were inoculated with *P. infestans* isolate 14-3-GFP and at 6 dai lesions were scored. The pictures in (A) were taken at 6 dai before (upper panel) and after (lower panel) trypan blue staining. The graph in (B) shows the percentage of inoculated sites that developed expanding lesions. The result is the average of three biological repeats (N≥27). Significant difference compared to the control is indicated by * (one-sided Student's t-test, $P<0.05$).

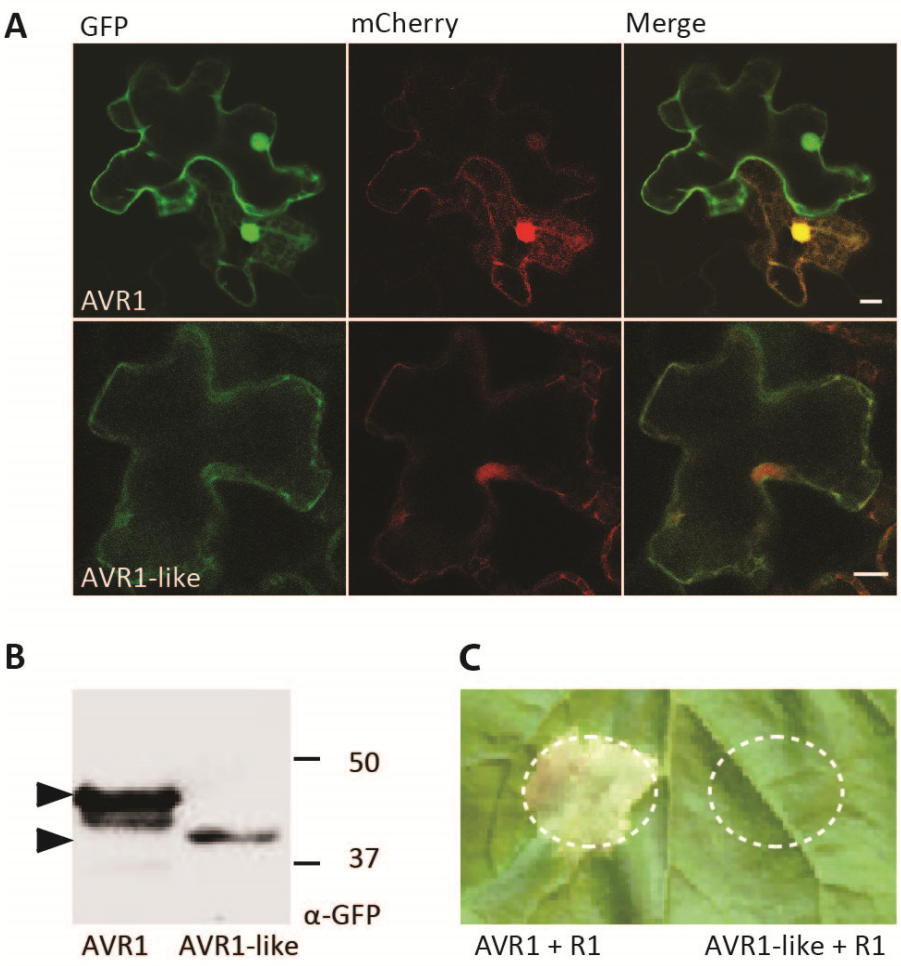

Figure 4. Transient expression of GFP-tagged AVR1 and AVR1-like in *N. benthamiana* leaves.

(A) AVR1 and AVR1-like localize in the nucleus and the cytoplasm. Confocal microscopy images showing the subcellular localization of AVR1 and AVR1-like. The *AVR1* or *AVR1-like* strain was co-infiltrated with a *mCherry* in a 1:1 ratio and a final OD of 0.1. Pictures taken at 2 dpi show cells co-transformed with *AVR1* or *AVR1-like* (green channel, left panels) and *mCherry* (red channel, middle panels). In the right panels the pictures are merged. Bar: 10 µm; c: cytoplasm; n: nucleus. (B) GFP-tagged AVR1 and AVR1-like proteins accumulate *in planta*. *AVR1* and *AVR1-like* were agroinfiltrated with an OD of 0.3. At 3 dpi proteins were extracted and subjected to SDS-PAGE and immunoblotting. Incubation with α-GFP shows the accumulation of GFP-tagged proteins indicated by black arrowheads. Molecular weight markers are indicated on the right. (C) AVR1, but not AVR1-like, elicits HR when co-expressed with R1. *AVR1* and *AVR1-like* were co-infiltrated with *R1* in a 1:1 ratio and a final OD of 0.5. Pictures were taken 3 dpi. Dotted lines indicate agro-infiltrated zones.

Nuclear localized AVR1 triggers R1-mediated HR

To test whether a specific subcellular localization of AVR1 is important for triggering R1-mediated HR, AVR1 was artificially targeted to the host cell nucleus or the host cell cytoplasm using the same targeting signals as described above for R1 (Figure S1). Confocal microscopy of leaves infiltrated with the various *AVR1* strains showed that AVR1^{NLS} is efficiently targeted to the nucleus, while AVR1^{NES} is almost completely excluded from the nucleus (Figure 5A). The control constructs AVR1^{nls} and AVR1^{nes} showed the same localization as AVR1-GFP (Figure 5A). To confirm that the fluorescence that we observed was indeed derived from the AVR1 fusion proteins we extracted the proteins and made a western blot. AVR1 fusion proteins were detected by probing the blot with GFP-antibodies and in all cases AVR1 fusion proteins of the expected size were present *in planta* (Figure 5B).

To determine which subcellular localization of AVR1 is required for triggering R1-mediated HR, we co-infiltrated the various *AVR1* strains with *R1* and monitored HR at 3 dpi. This revealed that the HR was less severe in leaves expressing AVR1^{NES} with AVR1 solely in the cytoplasm than in leaves expressing AVR1^{NLS} that targets AVR1 solely to the nucleus (Figure 5C). With AVR1^{NLS} more than 80% of the infiltrated sites showed strong HR compared to less than 40% and a weaker HR in the case of AVR1^{NES} (Figure 5D). Since cloning of AVR1 with a C-terminal NES failed we generated a second control construct for AVR1^{NES}, named AVR1^{nes*} which has the mutated NES peptide fused to the N-terminus of AVR1 (Figure S1). Similar to *AVR^{nes}*, *AVR1^{nes*}* triggers HR when co-infiltrated with *R1* (data not shown) and thus the presence of an extra peptide at the N-terminus of AVR1 does not abolish the function of AVR1. Therefore the severely reduced HR observed in leaves expressing AVR1^{NES} is most likely due to lack of AVR1 in the nucleus, and suggests that R1-mediated HR is only triggered in an efficient manner when AVR1 is localized in the nucleus.

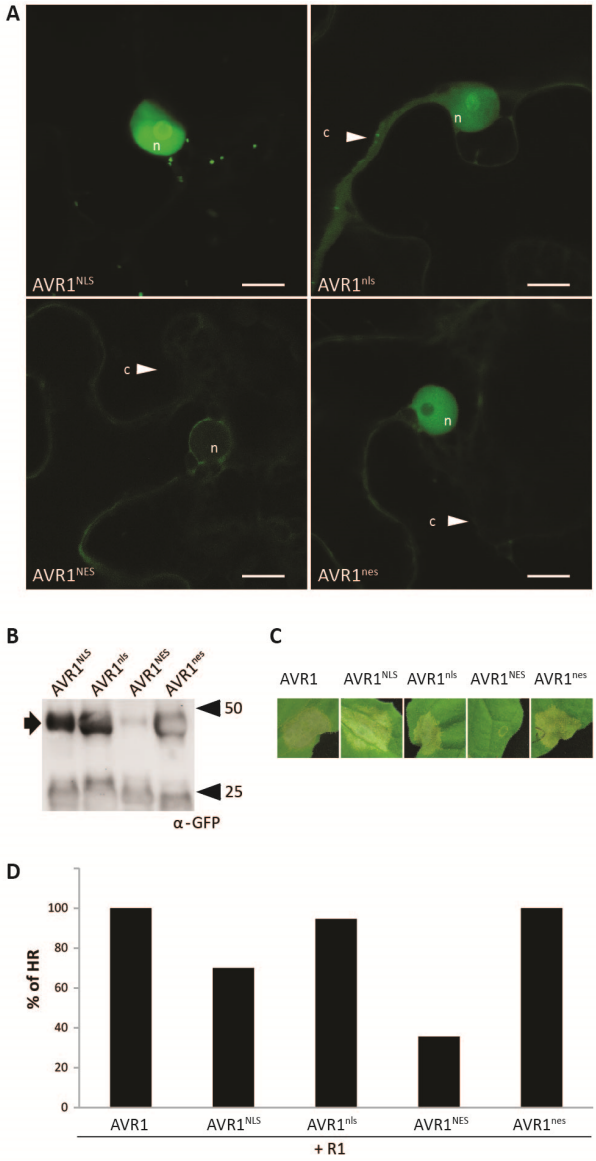

Figure 5. Transient expression of NLS- and NES-tagged AVR1 in *N. benthamiana* shows that nuclear localized AVR1 can elicit R1-mediated HR.
(A) Confocal microscopy images showing the subcellular localization of AVR1 modified with targeting signals NES and NLS and their mutant forms nes and nls. Strains carrying the fusion constructs were agroinfiltrated with an OD of 0.1 Pictures were taken at 2 dpi. Bars: 10 µm; c: cytoplasm; n: nucleus. (B) The AVR1 fusion proteins are stable *in planta*. The various *AVR1* strains were agroinfiltrated with an OD of 0.3. At 3 dpi proteins were extracted and subjected to SDS-PAGE and immunoblotting. Incubation with α-GFP shows the accumulation of the various AVR1 fusion proteins (black arrow). Molecular weight markers are indicated on the right. (C, D) Both nuclear and cytoplasmic targeted AVR1 partially lose the ability to elicit R1-mediated HR. *R1* was co-infiltrated with *AVR1*, *AVR1^{NLS}*, *AVR1^{nls}*, *AVR1^{NES}* or *AVR1^{nes}*, in a ratio of 1:1 and a final OD of 0.5. The pictures in (C) were taken at 3 dpi. The graph in (D) gives the number of infiltrated sites that show HR at 3 dpi out of a total of at least 30 per sample in two independent experiments, and expressed in percentages.

It thus seems that both AVR1 and R1 need to localize in the nucleus to activate immunity. To further confirm this, all possible combinations of strains containing the (modified) R1 and AVR1 constructs were co-infiltrated (Figure 6A) and the percentage of infiltrated sites showing HR was determined (Figure 6B). This clearly showed that the HR is only activated when both AVR1 and R1 are targeted to the nucleus.

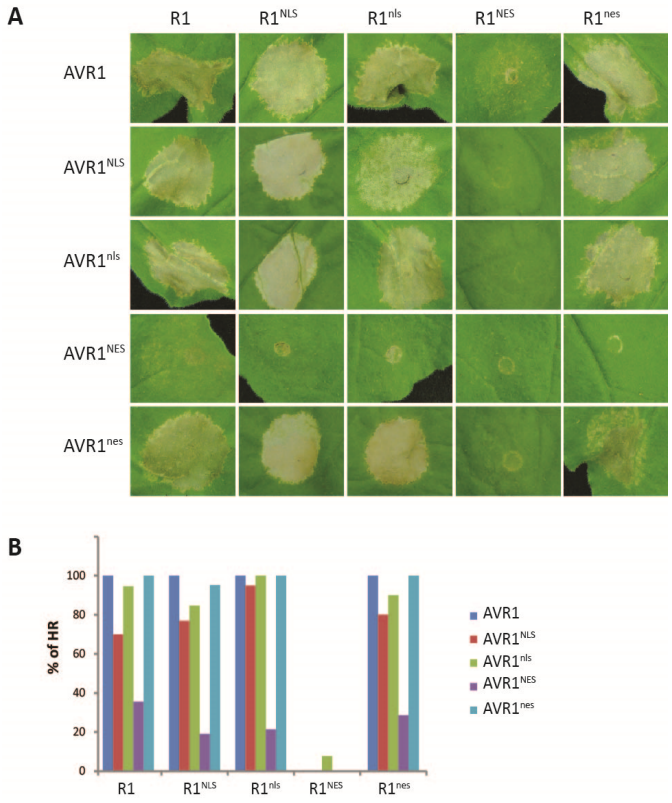

Figure 6. Transient co-expression of NLS- and NES-tagged R1 and AVR in *N. benthamiana* shows that nuclear localization of both R1 and AVR1 is required for HR.
The various combinations of *AVR1* and *R1* strains were co-infiltrated in a 1:1 ratio and a final OD of 0.5. Pictures in (A) were taken at 5 dpi. This experiment was repeated three times with similar results. The graph in (B) gives the average percentage of infiltrated sites showing HR from three biological replicates of each combination (N≥13).

Cytoplasmic localization of AVR1 is required for suppression of CRN2-induced cell death

Previously, we observed that AVR1 is able to suppress callose deposition and CRN2-induced cell death, properties that have been associated with potential virulence functions of RXLR effectors (Bouwmeester et al., 2011; Bos et al., 2010; Anderson et al., 2012). To investigate which subcellular localization is required for suppression of CRN2-induced cell death we co-expressed nuclear and cytoplasmic targeted AVR1 with CRN2 in *N. benthamiana* leaves. AVR1-GFP (strain *AVR1)* and GFP were used as positive and negative controls, respectively. As shown in Figure 7 AVR1^{NES} is able to suppress CRN2-induced cell death in 40% of the cases and this is more or less in a similar range as AVR1-GFP. In contrast, AVR1^{NLS} shows no suppression activity indicating that AVR1 has to be localized in the cytoplasm to suppress CRN2-induced cell death. The mutated version of NES, AVR1^{nes}, behaves as expected and similar to AVR1-GFP. However, the mutated version of NLS, i.e. AVR1^{nls}, shows no suppression of cell death. The mutation may not fully abolish NLS function and it could be that in the case of CRN2-induced cell death, the balance between nuclear and cytoplasmic localized AVR1 is much more sensitive

than in the case of R1-mediated HR. The latter is a very strong and rapid HR and the threshold to reach the effect is likely lower.

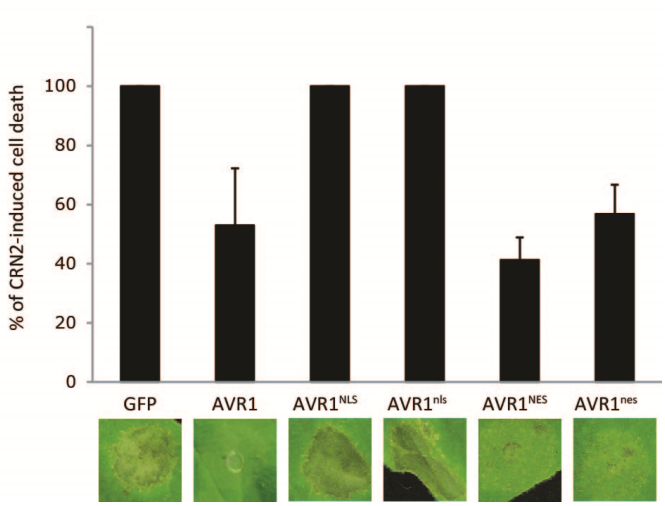

Figure 7. Cytoplasmic localization enables AVR1 to suppress CRN2-induced cell death.
Strains with NLS- and NES-tagged AVR1 constructs, and *GFP* and *AVR1* as negative and positive controls, respectively, were co-agroinfiltrated with *CRN2* in *N. benthamiana* leaves in a 2:1 ratio and a final OD of 0.6 for AVR1 and 0.3 for CRN2. Pictures were taken at 6 dpi. The graph gives the average percentage of infiltrated sites showing cell death at 6 dpi and based on 3 independent experiments (N≥29).

AVR1 needs a nucleocytoplasmic partitioning

When we co-expressed the various modified AVR1 constructs with the silencing suppressor P19 into *N. benthamiana* leaves, we observed that high expression of AVR1^{NES} but not AVR1^{NLS}, always resulted in a strong necrosis. Similar to AVR1^{NLS}, the mutated versions AVR1^{nls} and AVR1^{nes} never showed necrosis when transiently co-expressed with P19 (Figure 8A). To confirm that the necrosis induced by cytoplasmic localized AVR1 only occurs when co-expressed with P19, we infiltrated *GFP* or *AVR1^{NES}* with or without *P19* and measured ion leakage at 5 dpi as a way to quantify cell death. This indeed showed that AVR1^{NES} only triggers necrosis when co-expressed with P19. The GFP control does not trigger cell death irrespective of expression with or without P19 (Figure 8B). Because of the fact that cytoplasmic localization of AVR1 seems to be sufficient for its virulence functions, as shown by the suppression of CRN2-induced cell, one would anticipate that concentrating all AVR1 molecules in the cytoplasm would be ideal for *P. infestans* to promote infection. However, the strong necrosis in the *AVR1^{NES}* infiltrated leaves suggests that during infection AVR1 may also have a role in the nucleus, namely to avoid host cell death. Since AVR1-GFP (strain *AVR1)* without any modification does not trigger necrosis when co-expressed with P19, it seems that AVR1 requires a very balanced nucleocytoplasmic partitioning. The further

investigate this we tested if nuclear localized AVR1 is able to suppress the necrosis triggered by solely cytoplasmic localized AVR1. We co-infiltrated *P19* and *AVR1*^{NES} in combination with *GFP*, *AVR1*, *AVR1*^{NLS} or *AVR1*^{nls}. GFP had no effect and strong necrosis was observed in all cases. However, when AVR1 was targeted to the nucleus it could entirely suppress the necrosis inducing activity of AVR1^{NES} (Figure 8C) suggesting that nuclear localized AVR1 has a dominant negative effect on cell death triggered by cytoplasmic localized AVR1.

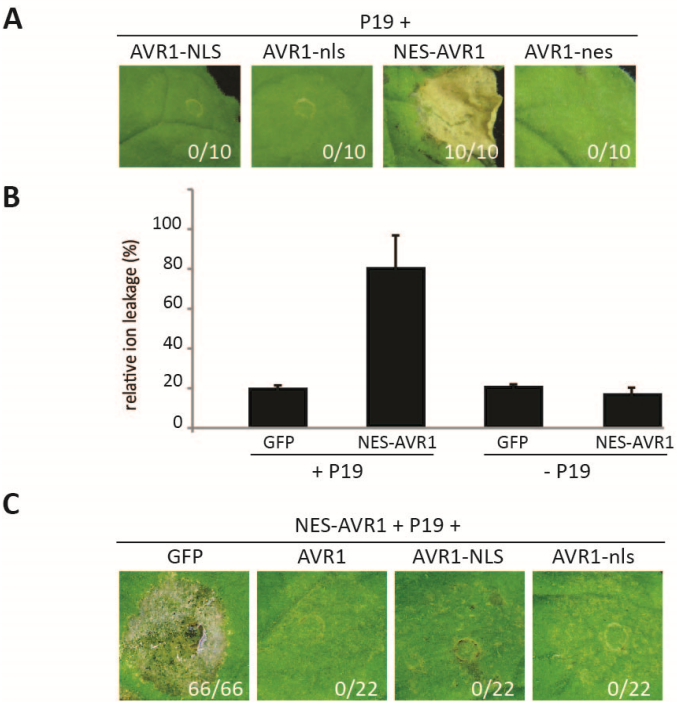

Figure 8. AVR1 needs a balanced nucleocytoplasmic partitioning for proper functionality.
(A and B) AVR1^{NES} triggers cell death when co-expressed with P19. Strains with NLS- and NES-tagged AVR1 constructs were co-agroinfiltrated with *P19* in *N. benthamiana* leaves in a 1:1 ratio and a final OD of 0.3. Pictures in (A) were taken at 5 dpi. The ratio indicates how many of the infiltrated sites show HR. (B) Quantification of cell death triggered by AVR1^{NES} in the presence (1:1 ratio; final OD 0.3) or absence (OD 0.3) of P19. GFP was included as control. The graph shows relative ion leakage values measured at 5 dpi. This experiment was repeated at least two times with similar results. (C) Nuclear localized AVR1 suppresses AVR1^{NES} triggered cell death. *AVR1*^{NES} and *P19* were infiltrated in *N. benthamiana* leaves together with *GFP*, *AVR1*^{NLS}, *AVR1*^{nls} or *AVR1* in in a 1:1:1 ratio and a final OD of 0.3. Pictures were taken at 5 dpi. The ratio indicates how many of the infiltrated sites show HR in a total of three independent experiments with similar results.

Discussion

In the 2002 paper of Ballvora et al. that described the cloning of the first late blight resistance gene, R1 was suggested to be anchored to the plasma membrane based on in silico prediction of four myristoylation sites (Ballvora et al., 2002). In this study we visualized the subcellular localization of R1 and its matching effector AVR1 by confocal microscopy and exploited subcellular targeting motifs to manipulate their localization, but found no evidence for R1 being localized at the plasma membrane. Instead we found that both, R1 and AVR1 localize to the nucleus as well as the cytoplasm and that immune activation (or ETI) requires a nuclear localization of the two matching partners. Immune suppression though, seems to be most effective when AVR1 is in the cytoplasm. The latter was measured by suppression of CRN2-induced cell death and in the absence of R1 while ETI was monitored by HR upon co-expression of R1 and AVR1 and also by lesion growth on leaves transiently expressing R1 and inoculated with a *P. infestans* isolate that has the *AVR1* gene (R1-mediated resistance).

The nucleocytoplasmic partitioning pattern that we observed for R1 is similar to that reported for several other plant NLR proteins (Shen et al., 2007; Tameling et al., 2010; Hoser et al., 2013; Inoue et al., 2013). However, the nuclear localization of R1 was not foreseen. According to a prediction by NLStradamus (Nguyen Ba et al., 2009; http://www.moseslab.csb.utoronto.ca/NLStradamus/; cut off value of 0.3) R1 has no significant NLS and it is a large protein (over 150 kDa) that cannot move by passive diffusion through the nuclear pores. Some nuclear localized NLRs, for example Arabidopsis RPS4 and SNC1 and tobacco N, contain a NLS in their sequence, but others, like barley MLA10 and potato Rx, do not (Wirthmueller et al., 2007; Cheng et al., 2009; Burch-Smith et al., 2007, Tameling et al., 2010; Shen et al., 2007). How R1 enters the nucleus is so far unknown. For MLA10 and Rx it has been found that they don't need a NLS to be imported into the nucleus. Rx is reported to use other means to translocate between the cytoplasm and nucleus (Tameling et al., 2010). In fact, Rx has to move in and out of the nucleus because it requires a balanced nucleocytoplasmic partitioning for activation of immunity (Tameling et al., 2010). MLA10 also seems to shuttle between the nucleus and the cytoplasm, and distinct functions for both nuclear and cytoplasmic-localized MLA10 have been reported (Bai et al., 2012). Similar to R1, also RPS4, SNC1, N and MLA10 were found to activate immunity inside the

nucleus. Here these NLRs may bind with transcription factors and in this way might regulate defense gene expression. This was shown for tobacco N and barley MLA10 (Burch-Smith et al., 2007; Shen et al., 2007; Padmanabhan and Dinesh-Kumar, 2014; Padmanabhan et al., 2013) and also, among others, for the Arabidopsis resistance proteins RCY1 and RRS1-R. RCY1 confers resistance to cucumber mosaic virus (CMV) and interacts via its CC-domain with a WRKY70 transcription factor that plays an important role in suppression of CMV multiplication (Ando et al., 2014). RRS1-R, that is active against bacterial wilt, contains a C-terminal WRKY DNA binding domain and functions inside the nucleus (Bernoux et al., 2008).

To show that the fluorescence patterns that we observed by confocal microscopy are indicative for localization of R1 or AVR1 and not due to free GFP cleaved from the fusion proteins we isolated proteins from agroinfiltrated leaves for western blot analysis. In this way we could detect the AVR1 fusion proteins (Figure 5B) but were unable to visualize the R1 fusion proteins. Apparently the R1 fusion proteins, which are relatively large, are unstable under the protein extraction conditions that we have used. Luckily we have other evidence showing that the R1 fusion proteins are stable *in planta*. With the exception of $R1^{NES}$ all R1 fusion proteins trigger HR in the presence of AVR1, indicating that the fusion proteins are functional. The fusion genes used in our assays are constructed in such a way that GFP is fused to the N-terminal end of R1 and the targeting signal to the C-terminal end. Since free GFP localizes to both nucleus and cytoplasm, we are quite sure that the nucleus-specific signals that we observe must represent the full-length protein.

The observation that nuclear localization of AVR1 is needed to trigger HR suggests that AVR1 needs to be in the close vicinity of R1. As yet we have no evidence for physical interaction between R1 and AVR1. For only a few R-AVR pairs there is solid evidence for direct protein-protein interaction, none of which concerns an oomycete RXLR avirulence effector. RXLR effectors are in general small proteins which might be able to passively diffuse into the nucleus through the nuclear pores. The mature AVR1 protein is 21 kDa in size and thus below the threshold of 40 kDa, the maximum size of proteins that can passively diffuse from cytoplasm to nucleus and back (Marfori et al., 2011). However, with the GFP-tag fused to AVR1, the fusion protein of 45 kDa just exceeds the threshold for passive diffusion and since AVR1 has no significant NLS (according to a prediction by

NLStradamus using a cut off value of 0.3) there might be some kind of carrier protein that mediates the transport over the membrane. So far there are only a few studies in which the localization of an RXLR effector was linked to its biological function. The plasma membrane localization of *Phytophthora sojae* Avh241 and *P. infestans* AVR2 was found to be critical for inducing plant cell death or triggering HR (Yu et al., 2012; Saunders et al., 2012). *P. infestans* RXLR effector PITG_03192 promotes infection when localized on the host ER membrane where it binds and prevents two NAC transcription factors from relocalization into the nucleus (McLellan et al., 2013). Avr3a localizes in the nucleus and the cytoplasm when expressed alone, but upon co-expression with R3a, the two together relocalize to vesicles. Inhibition of this relocalization by Wortmannin and BFA abolishes R3a/AVR3a-mediated HR (Engelhardt et al., 2012) indicating that relocalization of this R/AVR protein complex is essential for mediating immunity. In this study we have found that cytoplasmic localization of AVR1 is required for suppression of CRN2-induced cell death. However, when there is an overload of cytoplasmic AVR1 it leads to spontaneous cell death. This could be due to the fact that AVR1 stabilizes its host target SEC5 in the cytoplasm, which is a protein involved in exocytosis (**Chapter 4**). With too much AVR1 in the cytoplasm of the host cell, exocytosis might be blocked and this may trigger necrosis as a side effect. It thus seems that a balanced nucleocytoplasmic partitioning of AVR1 is needed for its biological functions, as this keeps AVR1 accumulation in the cytoplasm at a relative low level to avoid spontaneous cell death. It is also possible that AVR1, next to targeting Sec5 in the cytoplasm, has a function as virulence effector in the nucleus. In this regard it is interesting to note that there are two transcription factors among the putative host targets identified for AVR1 in an Y2H screen. In either case it would imply that transport of AVR1 between cell compartments is in one way or another regulated and not dependent on passive diffusion.

Similar to AVR1, the fungal avirulence factor AVR2 from *Fusarium oxysporum* and the bacterial avirulence factors PopP2 from *Ralstonia solanacearum* and AvrRPS4 from *Pseudomonas syringae* have to be present in the nucleus to activate NLR-mediated resistance (Ma et al., 2013; Heidrich et al., 2011; Bernoux et al., 2008). In the case of the tobacco mosaic virus protein p50 however, N-mediated HR in *N. benthamiana* is triggered when p50 is either solely localized in the cytoplasm or in the nucleus (Burch-Smith et al., 2007). This shows

that perception of pathogen effectors by matching NLS proteins can happen in both the host nucleus and cytoplasm. Our study demonstrated that despite the nucleocytoplasmic partitioning of AVR1 and R1, nuclear localization of the pair is required for R1-mediated HR. However, we cannot exclude the possibility that already in the cytoplasm AVR1 is recognized by R1 as its matching avirulence factor, while immunity is only activated in the nucleus. Thus far is not known how recognition in the cytoplasm is communicated to the nucleus. Hence, one of the next logical steps would be to determine what happens with R1 when it encounters AVR1 in the cytoplasm. Does it translocate to the nucleus and if so, is there a role for AVR1 in the actual translocation or do they translocate together.

Materials and methods

Plasmids construction

Primer pairs that were used for amplifying the full length *AVR1* or *R1* gene are shown in Table 1. For fusing NLS/nls or NES/nes peptides to AVR1 and R1, the forward or reverse primers was extended with sequences encoding the NLS/nls (Haasen et al., 1999) or NES/nes (Wen et al., 1995) peptides (Table S1). The PCR amplicons were cloned into pENTR/D-TOPO entry vector (Invitrogen) and subsequently recombined into the binary vector pSOL2094. The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain AGL1. Figure S1 shows cartoons of all the constructs used in this study.

Agroinfiltration in *N. benthamiana*

Plants were grown in a greenhouse under standardized conditions for 5 weeks. For agroinfiltration we basically followed the procedures described by van der Hoorn et al. (2000) and Champouret et al. (2009). *A. tumefaciens* strains harbouring the binary vectors were cultured in YEB medium with appropriate antibiotics for 18 hours at 28°C. The cells were collected by centrifugation, resuspended in agroinfiltration medium and adjusted to the desired concentration. For co-expression, *Agrobacterium* strains were mixed in a 1:1 ratio. Agroinfiltrated plants were kept in a climate chamber at 25°C with a 12 h photoperiod and a 70% relative humidity (RH).

Infection assays

Detached leaf assays were performed as previously described (Vleeshouwers et al., 1999). Zoospores were obtained from 10 day old cultures of *P. infestans* isolates 14-3-GFP and 88069 grown on rye sucrose agar medium at 18°C the zoospore suspension was adjusted to a concentration of $2*10^4$ mL^{-1}. Leaves detached from 5 week old *N. benthamiana* plants were inoculated on the abaxial side with 10 µl droplets of the suspension and incubated at high humidity at 18°C, with the first 24 hours in the dark. Lesion sizes were determined at 6 dai.

Confocal microscopy

At 2 dpi, patches were cut from *N. benthamiana* leaves and used for confocal imaging on a Zeiss LSM 510-META 18 confocal laser scanning microscope. Excitation of GFP and mCherry fluorescence was performed using an Argon laser (488 nm), and a HeNe 1 laser (543 nm), respectively. Fluorescence was captured by 505-530 nm (GFP) or 600-650 nm (mCherry) filters. LSM software was used for data processing.

Ion leakage measurements and staining

Ion leakage measurement and trypan blue staining were performed as previously described (Bouwmeester et al., 2014; Bouwmeester et al., 2011).

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Supplementary information

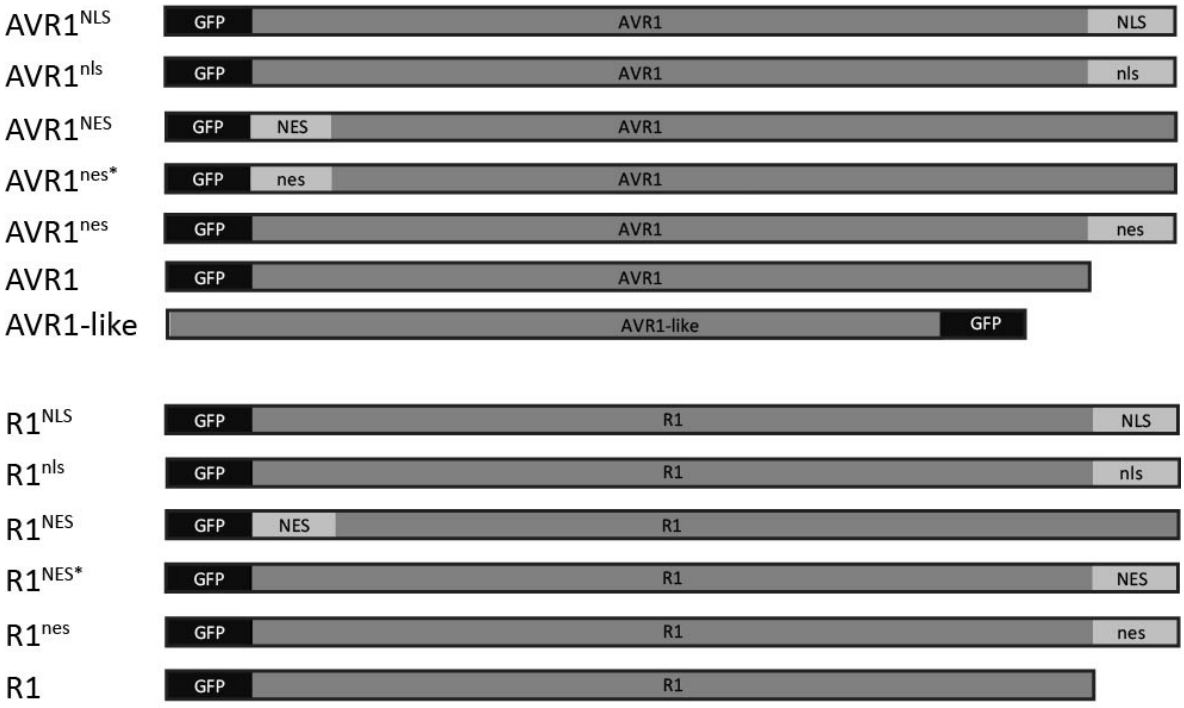

Figure S1. Cartoons showing all the constructs used in this study.
The AVR1 constructs contain 555 nucleotides coding sequence of the *AVR1* gene that covers the RXLR motif and the C-terminal effector domain but lacks the signal peptide and stop codon. The AVR1-like construct contains 441 nucleotides coding sequence of the *AVR1-like* gene that covers the RXLR motif and the C-terminal effector domain but lacks the signal peptide and stop codon. The R1 constructs contain 3879 nucleotides coding sequence of the *R1* gene covering the full length R1 protein without stop codon.

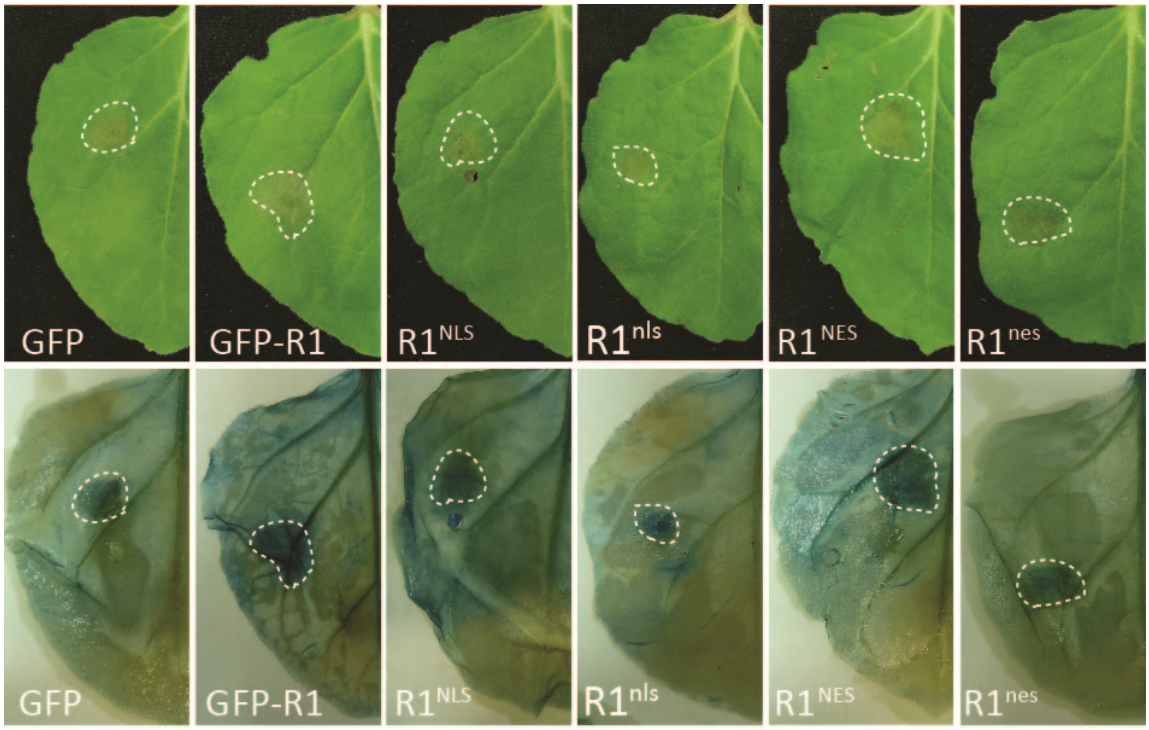

Figure S2. *N. benthamiana* leaves expressing R1 do not confer resistance to *P. infestans* isolate 88069. Subcellular targeted R1 was transiently expressed in *N. benthamiana* leaves (OD 0.3). GFP and R1 were included as control. At 1 dpi the leaves were inoculated with *P. infestans* 88069, an isolate lacking the *AVR1* gene. At 6 dai lesions development were scored. Pictures were taken before (top) and after (bottom) trypan blue staining.

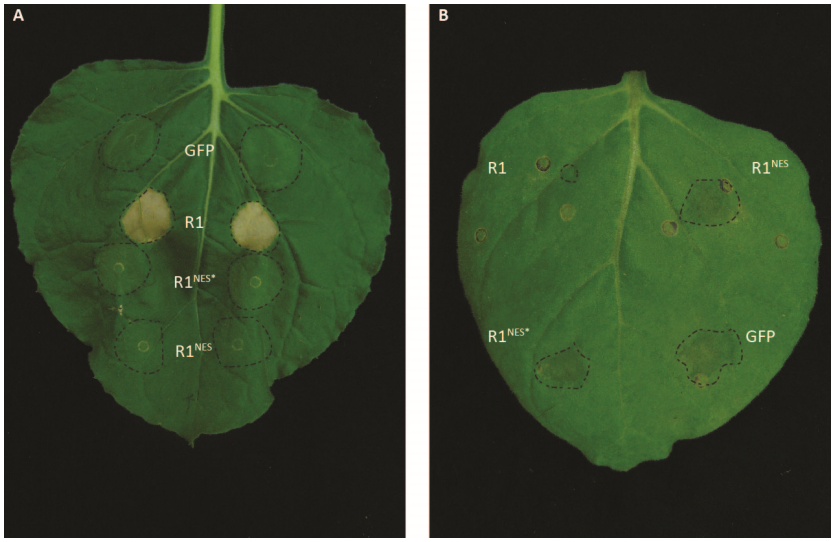

Figure S3. Cytoplasmic targeted R1 loses its function as immune receptor.
(A) Strains with R1 constructs in which NES is fused to either the N-terminus (*R1*^{NES}) or the C-terminus (*R1*^{NES*}) were co-agroinfiltrated with *AVR1* in *N. benthamiana* leaves in a 1:1 ratio and a final OD of 0.3. *GFP* and *R1* were included as negative and positive control, respectively. The picture was taken at 6 dpi and is representative for the results obtained in ≥ 20 infiltrated sites per construct. Dotted lines indicate the infiltration zone. (B) Lesion development on a *N. benthamiana* leaf inoculated with *P. infestans* isolate 14-3-GFP. The leaf was agroinfiltrated with *R1*, *R1*^{NES}, *R1*^{NES*} or *GFP* one day before inoculation. The picture was taken at 6 dai and is representative for the results obtained in ≥ 15 infiltrated and inoculated sites per construct. Dotted lines indicate the lesion area.

Table S1. Primers used in this study.

Primer name	Sequence (5' - 3')
R1-F	caccatgaatttcaacaatgaattgtctgatctg
R1-Rwo	tcttatttctgcaagaatatttttttactgc
R1^{NES*}-F	caccatgaacgagcttgcattaaagctcgctggtcttgatattaacaagaatttcaacaatgaattgtctgatctg
R1^{NES}-R	cttgttaatatcaagaccagcgagctttaatgcaagctcgtttcttatttctgcaagaatatttttttactgc
R1^{nes}-R	cttgttagcatctgcaccagctgcctttaatgcaagctcgtttcttatttctgcaagaatatttttttactgc
R1^{NLS}-R	agggtcctcaaccttacgcttcttttttaggtcttatttctgcaagaatatttttttactgc
R1^{nls}-R	agggtcctcaaccttacgcttgtttttaggtcttatttctgcaagaatatttttttactgc
AVR1-F	caccatgttcgaccacgacaagg
AVR1-Rwo	aaatggtaccacaacatgtccaccaagc
AVR1^{NES}-F	caccatgaacgagcttgcattaaagctcgctggtcttgatattaacaagttcgaccacgacaaggttc
AVR1^{nes*}-F	caccatgaacgagcttgcattaaaggcagctggtgcagatgctaacaagttcgaccacgacaaggttc
Avr1^{nes}-R	cttgttagcatctgcaccagctgcctttaatgcaagctcgttaaatggtaccacaacatgtccaccaagc
Avr1^{NLS}-R	agggtcctcaaccttacgcttcttttttaggaaatggtaccacaacatgtccaccaagc
Avr1^{nls}-R	agggtcctcaaccttacgcttgtttttaggaaatggtaccacaacatgtccaccaagc
AVR1-like-F	caccatggccggattcgaccac
AVR1-like-Rwo	aaatggtaccacaacatgtccacc

Chapter 4

Phytophthora infestans RXLR effector AVR1 interacts with exocyst component Sec5 to manipulate plant immunity

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Abstract

Phytophthora infestans secretes a significant number of effectors that modulate host defense and thereby pave the way for successful infection and colonization. Mechanisms by which effectors modulate host defense are largely unknown. AVR1 is an RXLR effector from *P. infestans* that is detected by potato resistance protein R1 leading to R1-mediated resistance. In the absence of R1, AVR1 can suppress pathogen-associated molecular pattern-triggered immunity (PTI) like callose deposition. To investigate the mechanism of AVR1-modulated host defense, a yeast-2-hybrid screening was performed to search for plant proteins interacting with AVR1. One of the candidates is Sec5, which is a subunit of the exocyst, a protein complex involved in vesicle trafficking. Characterization of the Sec5-AVR1 interaction *in planta* revealed that the two are in close proximity and that AVR1 stabilises and sequesters Sec5. Sec5 is required for secretion of the pathogenesis-related protein PR-1 and for callose deposition, which indicates its role in exocytosis and plant defense. Sec5 plays a role in R1-mediated resistance to *P. infestans* suggesting a role in innate immunity independent from exocytosis. Our findings show that *P. infestans* manipulates a protein that is involved in host vesicle trafficking and thereby potentially disturbs focal secretion. This is a novel strategy that oomycete pathogens exploit to modulate host defense.

Introduction

Phytophthora species are oomycete plant pathogens that are widespread and able to infect a vast group of plants including many economically important crops. To date over 100 *Phytophthora* species have been described, each with their own specific narrow or wide host range (Kroon et al., 2012). The most infamous species is *Phytophthora infestans*, the late blight pathogen that was responsible for the Irish potato famine in the 1840s and to date, still causes great losses in potato and tomato production worldwide. *P. infestans* is a hemibiotrophic pathogen. To establish infection and subsequent colonization of host tissue, *P. infestans* secretes effectors that modify and manipulate the host cell machinery to suppress defense responses. A better insight into how pathogen effectors modulate host defense would help in designing more targeted strategies towards improving disease resistance in crops.

P. infestans has a remarkable large number of genes encoding secreted effector proteins, which can be roughly divided into two classes (Haas et al., 2009; Stassen and Van den Ackerveken, 2011). One class encodes extracellular effectors such as enzyme inhibitors, which counteract hydrolytic enzymes secreted by the host to block host defense, or cell death elicitors including elicitins (e.g. INF1) and NEP-like proteins (NLPs). The other class encodes effectors that mostly exert their activity inside host cells and are thus often named intracellular effectors. These comprise so-called RXLR effectors and Crinklers (CRNs), proteins with short conserved motifs in their N-terminus that are required for translocation into host cells. In RXLR effectors this motif is arginine-X-leucine-arginine (RXLR) often followed by an EER motif (i.e. two glutamic acid residues and an arginine). Genome sequencing revealed that the 240 Mb genome of *P. infestans* contains ~560 RXLR effector genes and over 180 *CRN* genes (Haas et al., 2009), and this is significantly more than in any other oomycete sequenced to date. Emerging evidence indicates that RXLR effectors have dual activities. On the one hand, RXLR effectors are able to suppress defense responses in the plant (Bos et al., 2006; Oh et al., 2009; Halterman et al., 2010), in particular PAMP-triggered immunity (PTI) elicited by pathogen-associated or microbe-associated molecular patterns (PAMPs/MAMPs) (Jones and Dangl, 2006). On the other hand, RXLR effectors are able to activate plant defense responses. This so-called effector-triggered immunity (ETI) (Jones and Dangl, 2006; Hardham and Cahill, 2010) is often a very strong response reminiscent of a hypersensitive response (HR) and

leading to local cell death. This response only occurs when the plant has a resistance (R) protein that specifically recognizes one of the RXLR effectors. In fact, the conserved RXLR motif was first discovered in a set of diverse secreted oomycete proteins that shared the ability to elicit an effective defense response in plants carrying specific *R* genes (Rehmany et al., 2005). This phenomenon, now named ETI, was previously known as a resistance response governed by a gene-for-gene interaction with a repertoire of avirulence (*AVR*) genes in the pathogen and corresponding *R* genes in the host. In *P. infestans* a number of RXLR effectors, including AVR1, AVR2, AVR3a, AVR4, IPI-O1 (is AVR-blb1), AVR-blb2 and AVR-vnt1, have been demonstrated to act as avirulence factors according to this classical gene-to-gene model (Vleeshouwers et al., 2011) and in most cases their corresponding *R* genes have been identified. The first one to be cloned was *R1* (Ballvora et al., 2002) and, similar to *R1*, all late blight *R* genes identified thereafter encode intracellular nucleotide-binding domain / leucine-rich repeat (NLR) proteins (Vleeshouwers et al., 2011). Despite the fact that the tem avirulence factor has become obsolete, the nomenclature of several RXLR effector genes still refers to the original concept of the gene-for-gene model.

This study deals with *AVR1*, a *P. infestans* gene identified by map-based cloning and marker landing, and based on its ability to elicit an HR when co-expressed with *R1* in *Nicotiana benthamiana* (Guo, 2008). *AVR1* encodes a 208 amino acid protein consisting of a signal peptide, an RXLR-dEER domain with the motif RQLR and DEAR, and a C-terminal region that determines its effector activity. Similar to other RXLR effectors the C-terminal region of AVR1 lacks homology to any other protein but has two W motifs and one Y motif, two of the three conserved motifs that have been identified by bioinformatic analysis of over 700 RXLR effectors (Jiang et al., 2008). *P. infestans* isolates that are virulent on *R1* potato plants have a deletion at the *AVR1* locus but possess a homologous gene (named *AVR1-like*) at another locus. At the protein level AVR1-like shows about 82% homology to AVR1 but due to a premature stop codon there is a 38 amino acid truncation at its C-terminus. Despite the relatively high homology with AVR1 and the conservation of the W and Y motifs, AVR1-like is not able to trigger R1 mediated resistance on potato plants.

Plants have developed multiple defense strategies to inhibit pathogen invasion. It is obvious that the secretory pathway is involved in defense because of the secretion of antimicrobial compounds to the apoplast (Robatzek, 2007) and

there is increasing evidence that vesicle trafficking is important for an effective immune response in plants. For example, the plasma membrane-associated syntaxins NbSYP132 and PEN1 (or SYP121) known as SNAREs, were reported to be required for accumulation of the pathogenesis-related protein PR-1 in the apoplast and for assembly of papillae and focal secretion, respectively (Kalde et al., 2007; Collins et al., 2003). One step upstream of SNAREs is the exocyst that tethers post-Golgi vesicles to the plasma membrane (He and Guo, 2009). The exocyst, which was initially discovered in yeast, is a protein complex consisting of eight subunits (Hsu et al., 1996; TerBush et al., 1996). Its function is extensively studied in yeast and animals (Hsu et al., 2004). In contrast, the plant exocyst is hardly studied but is known to be essential for plant development and involved in polarized secretion and exocytosis (Hala et al., 2008; Wen et al., 2005; Cole et al., 2005; Synek et al., 2006). More recent reports point to roles in other processes including plant defense. The exocyst subunit Exo70B2, for example, was shown to be important for cell wall apposition formation and PTI (Pecenkova et al., 2011; Stegmann et al., 2012). An anticipated role of vesicle trafficking and the exocyst in defense is supported by the observation that effectors secreted by bacterial and fungal plant pathogens target the vesicle trafficking machinery in the host to facilitate colonization. One example is the *Pseudomonas syringae* effector HopM1 which destabilises *Arabidopsis* AtMIN7, an adenosine diphosphate (ADP) ribosylation factor (ARF) guanine nucleotide exchange factor (GEF), via the host proteosome (Nomura et al., 2011; Nomura et al., 2006). This ARF-GEF protein is a key regulator of vesicle formation and its destabilization leads to enhanced pathogen colonization. *P. syringae* effector HopZ2 targets *Arabidopsis* MLO2 to suppress PEN1-dependent secretion of defense components (Lewis et al., 2012). Another example is WtsE, an AvrE-family type III effector secreted by the corn pathogen *Pantoea stewartii* subsp. *stewartii.* This effector carries a putative ER membrane localization signal that is important for its virulence function such as forming water-soaked lesions and induction of necrosis (Ham et al., 2006). The fungal plant pathogen *Alternaria carthami* secretes the phytotoxin Brefeldin A (BFA) that inhibits formation of Golgi-derived vesicles (Driouich et al., 1997). BFA impedes *Arabidopsis* penetration resistance by blocking callose deposition and the accumulation of syntaxin PENETRATION (PEN)1 at the plasma membrane (Nielsen et al., 2012).

To date a number of RXLR effectors have been exploited to study the mechanisms by which oomycetes manipulate host immunity, and results show that these mechanisms vary. *Phytophthora sojae* Avh331 and *P. infestans* PexRD2 were found to disturb or target MAPK signalling, a pathway important for plant defense (Cheng et al., 2012; King et al., 2014), while *P. sojae* AVR3b, that has NADH and ADP-ribose pyrophosphorylase activity, manipulates host immunity by interfering with ETI (Dong et al., 2011). *Phytophthora parasitica* RXLR effector PSE-1 (penetration-specific effector 1) promotes colonization by impeding with host auxin physiology (Evangelisti et al., 2013). *P. infestans* RXLR effector IPI-O1 disrupts the integrity of adhesions between plasma-membrane and cell wall and uses the plasma membrane spanning lectin receptor kinase LecRK-I.9 as a host target (Bouwmeester et al., 2011). *P. infestans* AVR3a^{KI} is able to stabilize the host E3 ligase CMPG1 to modulate the host immunity (Bos et al., 2010). *P. infestans* Pi03192 hampers the re-localization of two host transcription factors to promote pathogen colonization (McLellan et al., 2013). *P. infestans* AVRblb2 is able to inhibit the secretion of the plant immune protease C14 to the haustorial interface (Bozkurt et al., 2011), indicating that effectors can have the ability to interfere with plant focal secretion. However, as yet there are no reports about oomycete pathogen effectors that specifically target the vesicle trafficking machinery to modulate the host immune system.

The aim of this study was to unravel the mechanism by which AVR1 manipulates host defense. Yeast two hybrid assays were carried out to identify host proteins interacting with AVR1. One of the candidates selected as a potential host target of AVR1 was the exocyst component Sec5. Characterization of the Sec5-AVR1 interaction *in planta* revealed that AVR1 stabilises and sequesters Sec5. Sec5 is specifically involved in *R1*-mediated resistance to *P. infestans* suggesting a role in innate immunity independent from exocytosis. Altogether, these findings point to a novel strategy that oomycete pathogens exploit to modulate host defense, and that is modulating host focal secretion by manipulating proteins that are part of the vesicle trafficking machinery.

Results

P. infestans RXLR effector AVR1 interacts with plant exocyst component Sec5

Yeast two-hybrid (Y2H) screenings that were performed to identify host targets of *P. infestans* RXLR effector AVR1, resulted in a set of clones coding for potato proteins potentially interacting with AVR1. The set included three clones encoding the exocyst component Sec5 with one (named StSec5-a) harbouring an insert representing an almost full length *Sec5* cDNA sequence; the encoded protein lacks only three amino acids at the N terminus (Figure S1). Using gene-specific primers (Table S1) a full length potato *Sec5* cDNA clone (StSec5) was obtained. In order to investigate the interaction specificity between Sec5 and AVR1 in Y2H assays, we co-transformed the prey plasmid containing *StSec5* with bait plasmids containing either *AVR1*, *AVR1-like*, *IPI-O1* or *IPI-O4.* This confirmed the Sec5-Avr1 interaction in yeast and showed that this interaction is specific; only AVR1 interacts with Sec5 and none of the other three RXLR effectors, i.e. AVR1-like, IPI-O1 and IPI-O4 (Figure 1A). Co-transformation of the nearly full length and the partial *StSec5* clones with the four RXLR effectors confirmed that Sec5 specifically interacts with AVR1 (Figure S2).

To investigate whether AVR1 and Sec5 interact *in planta*, full length StSec5 with a N-terminal GFP-tag (GFP-StSec5) and AVR1 with a C-terminal myc-tag (AVR1-myc) were co-expressed in *Nicotiana benthamiana* leaves. Co-immunoprecipitation (Co-IP) was performed 3 day post infiltration (3 dpi). As shown in Figure 1B, AVR1-myc co-immunoprecipitates with GFP-StSec5, while AVR1-like-myc does not (Figure 1B). Moreover, in the absence of GFP-StSec5, AVR1-myc is not co-immunoprecipitated by anti-GFP conjugated agarose demonstrating that AVR1 is specifically associated with a complex that contains StSec5 (Figure 1B). Taken together these results show that AVR1 associates with Sec5 *in planta*.

Phylogenetic analysis of Sec5

Sec5 is one of the eight subunits of the exocyst complex. This complex functions in vesicle trafficking, a process that plays an essential role in eukaryotes. Consequently, exocyst subunits are conserved proteins that can be found throughout the eukaryotic kingdoms (Zhang et al., 2010; Elias et al., 2003; Terbush and Novick, 1995; TerBush et al., 1996; Hsu et al., 1996). However,

unlike yeast and animals that contain single copy genes for each exocyst subunit, plants have multiple genes for over half of the exocyst subunits (Elias et al., 2003; Synek et al., 2006; Zhang et al., 2010). Arabidopsis for example, has two *Sec5* genes, *Sec5A* and *Sec5B* (Chong et al., 2010). To investigate the Sec5 distribution in *Solanaceous* plants we mined the Sol Genomics Network database for Sec5 and compared the deduced protein sequences with known Sec5 sequences from plants, animals and yeast (Chong et al., 2010). Phylogenetic analysis showed two distinct clades, one containing plant Sec5 proteins and the other grouping the animal Sec5's (Figure S4). Tomato and potato have each two nearly identical Sec5 proteins whereas *N. benthamiana* has four Sec5 proteins, which is likely due to its allopolyploid genome. The *Solanaceous* Sec5 proteins clustered together into two subgroups and their phylogenetic relationship suggests a gene duplication event in the last common ancestor of *Solanaceous* plants.

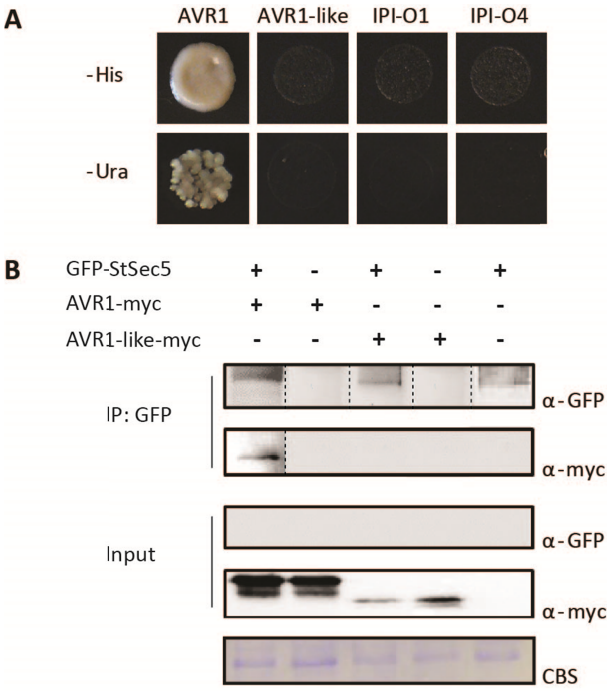

Figure 1. *P. infestans* RXLR effector AVR1 specifically associates with Sec5.
(A) *P. infestans* AVR1 interacts with potato Sec5 (StSec5) in yeast. Co-transformation of yeast with a Y2H prey vector containing Sec5 and a Y2H bait vector containing AVR1, AVR1-like, IPI-O1 or IPI-O4 as indicated. Only co-expression of Sec5 and Avr1 resulted in growth on medium lacking His [-His] or Ura [-Ura].(B) Co-immunoprecipitation showing that AVR1 interacts with StSec5 *in planta*. Total protein extracts (input) of *N. benthamiana* leaves infiltrated with the indicated constructs (+), and protein complexes immunoprecipitated with GFP-trap_A® beads (IP: GFP) were separated on gels and blotted. GFP- and myc-tagged fusion proteins were detected by probing western blots with α-GFP and α-myc. Only AVR1 is detected in a complex with StSec5. CBS: Coomassie Blue Staining of the blot containing total protein extracts showed equal loading in each lane based on the 50 kDa RuBisCo band.

AVR1 stabilizes Sec5 *in planta*

In the western blot analyses that were performed to demonstrate AVR1-Sec5 interaction *in planta* (Figure 1B) GFP-StSec5 was only detected after immunoprecipitation with anti-GFP conjugated agarose and not in the total protein extracts. This suggested that GFP-Sec5 has low steady state levels under the experimental conditions used. To investigate whether AVR1 alters the steady levels of StSec5 *in planta*, StSec5 was co-expressed with either AVR1-myc, AVR1-like-myc or empty vector (EV) in *N. benthamiana* leaves. Leaf samples were collected at 2, 3 and 5 dpi and protein extracts were analysed before and after immunoprecipitation. The results show that after immunoprecipitation with anti-GFP conjugated agarose StSec5 can be detected in all samples but that the amounts vary. In the presence of AVR1, there is more StSec5 detectable than in the presence of Avr1-like or in the EV control (Figure 2A), and this suggests that AVR1, but not AVR1-like, affects the accumulation of StSec5. In a similar experiment we co-expressed a GFP-HA construct with AVR1, AVR1-like or EV and observed that levels of accumulated GFP-HA were the same, irrespective of the presence of AVR1 or AVR1-like, or in the EV control (Figure S3). Apparently AVR1 specifically stabilizes StSec5.

To find additional support for the *in planta* interaction between Sec5 and AVR1, we performed bimolecular fluorescence complementation (BiFC). N- or C-terminal portions of Yellow Fluorescent Protein (YFP) coding sequences were fused to *AVR1*, *AVR1-like*, *AVR2* or *StSec5*, and constructs that harbour complementary portions of YFP were co-expressed in *N. benthamiana* leaves. Confocal microscopy revealed that a combination of AVR1:YC and YN:StSec5 restored YFP resulting in fluorescence (Figure 2B). This confirms that *in planta* AVR1 and StSec5 are in the close proximity. In contrast, no YFP fluorescence was observed when AVR1-like:YC or YC:AVR2 was co-expressed with YN:StSec5 again demonstrating specificity between Sec5 and AVR1.

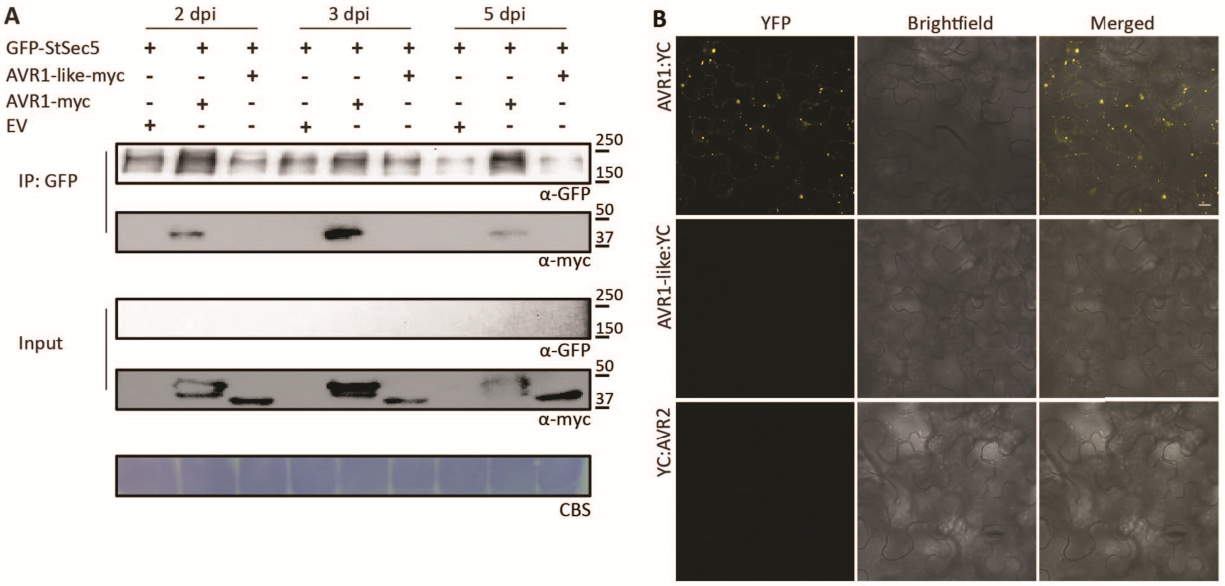

Figure 2. AVR1 stabilizes Sec5.
(A) Co-expression with AVR1 affects the steady state levels of StSec5. Total protein extracts (input) of *N. benthamiana* leaves 2, 3 and 5 dpi with the indicated constructs (+), and protein complexes immunoprecipitated with GFP-trap_A® beads (IP: GFP) were separated on gels and blotted. GFP- and myc-tagged fusion proteins were detected by probing western blots with α-GFP and α-myc. The result is representative for three independent experiments. CBS: Coomassie Blue Staining of the blot containing total protein extracts showed equal loading in each lane based on the 50 kDa RuBisCo band. (B) BiFC confirms that AVR1 interacts with StSec5. The C-terminus of YFP (YC) was fused to the C-terminus of AVR1, AVR1-like and AVR2, and the N-terminus (YN) of YFP was fused to the N-terminus of StSec5. YN:StSec5 was co-expressed with AVR1:YC, AVR1-like:YC and AVR2:YC in *N. benthamiana* leaves. Images were taken using confocal microscopy at 2 days post infiltration (dpi). Bar = 10 μm.

Sec5-silencing reduces plant growth and alters leaf development

To study the role of Sec5 in plant defense, we set out to perform functional assays in leaves with reduced levels of *Sec5*. For this purpose we exploited virus-induced gene silencing (VIGS). Since *Sec5* genes in *Solanaceous* plants are highly conserved, we generated a VIGS construct that targets *Sec5* in both *N. benthamiana* and tomato (Figure S5). As shown in Figure 3A, silencing of *Sec5* altered plant growth of *N. benthamiana* (Figure 3A). *Sec5*-silenced plants (TRV:*Sec5*) were smaller when compared to control plants (TRV:*GUS*); in particular the first six leaves were smaller and thicker while the upper younger leaves were small and curved. Also in tomato, *Sec5*-silenced plants were smaller

than the control plants (Figure S6A). Q-RT-PCR analyses showed strongly reduced levels of *Sec5* mRNA to on average 16% of normal levels in *N. benthamiana* (Figure 3B) and 36% of normal levels in tomato (Figure S6B). As a control we also silenced *SGT1,* a gene encoding a chaperone protein which is required for NLR-mediated resistance. This resulted in shorter and more branched plants (Figure 3A), similar to the phenotype described previously (Peart et al., 2002). Taken together these results demonstrate that *Sec5*-silencing is effective and point to an important role for Sec5 in plant development.

Sec5 is required for full R1/AVR1 HR

To address the question whether Sec5 is required for HR elicited by AVR1 in the presence of R1, we co-expressed R1 and AVR1 in *Sec5*-silenced *N. benthamiana* plants. Already at 2 days after agroinfiltration (2 dpi) of the R1 and AVR1 constructs clear cell death responses reminiscent of HR were observed in control plants (TRV:*GUS*) but no responses were visible on *SGT1*-silenced plants (TRV:*SGT1*) nor in *Sec5*-silenced plants (TRV:*Sec5*). At 6 dpi the rate of HR was scored and pictures were taken (Figure 3C-E). By that time all R1/AVR1 infiltrated sites on the control plants showed HR (100%). Also on *SGT1*-silenced and *Sec5*-silenced plants some responses were visible, but in comparison to the control plants the percentage of infiltrated sites that showed HR was significantly reduced to less than 15% on TRV:*SGT1* and ~60% on TRV:*Sec5* plants (Figure 3E). A similar reduction was observed on *Sec5*-silenced tomato plants (Figure S6C and 6D). To investigate to what extent silencing of *Sec5* affects HR in general, we tested two other R/AVR pairs, i.e. the potato NLR protein R3a with its cognate *P. infestans* RXLR effector AVR3a (Armstrong et al., 2005) and the potato NLR protein Rx with the coat protein (CP) of Potato virus X (Tameling et al., 2010). Responses were scored in a similar manner at 6 dpi. On control plants all infiltrated sites showed HR and this was more or less similar on *Sec5*-silenced plants (Figure 3F). On *SGT1*-silenced plants a significant reduction was observed for both R/AVR pairs and this is consistent with previous reports (Bos et al., 2006; Azevedo et al., 2006). In conclusion, these results show that Sec5 is required for HR triggered by R1/AVR1 but not necessarily for HR triggered by other R/AVR pairs.

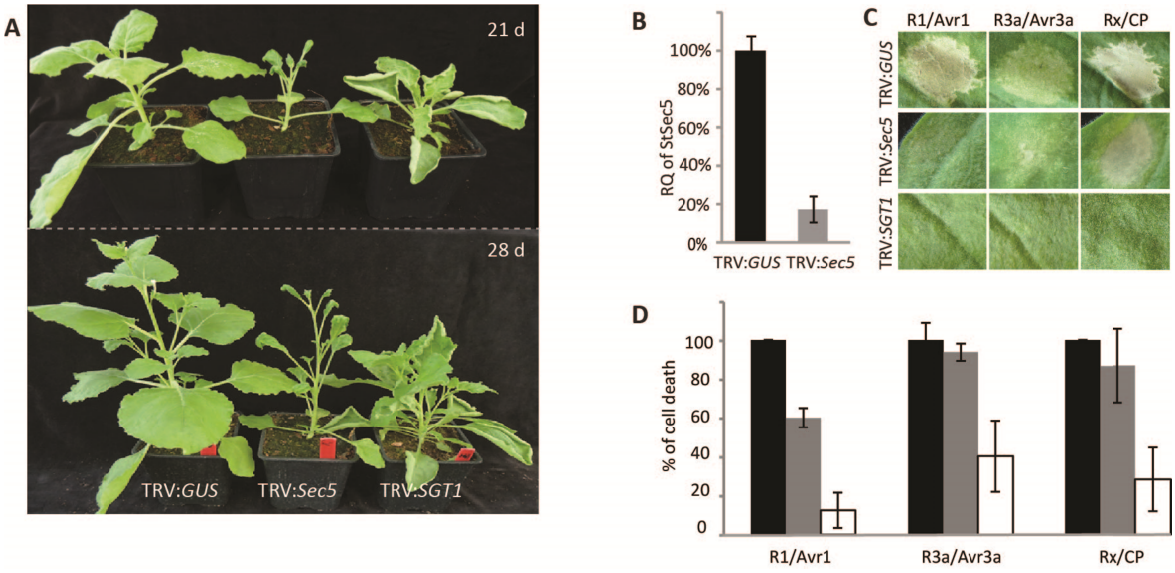

Figure 3. Sec5 is involved in R1 recognition of AVR1.
(A) Phenotypes of *N. benthamiana* plants at 21 and 28 days after inoculation with TRV:*GUS* (control) and the silencing constructs TRV:*Sec5* and TRV:*SGT1*. (B) *Sec5* expression in *N. benthamiana* plants three weeks after inoculation with the TRV:*Sec5* silencing construct. Q-RT-PCR analysis was performed using *Actin* expression for normalization. On the Y-axis the Relative Quantification (RQ) of *Sec5* expression is shown with the expression in the control (TRV:*GUS*) plants set at 100%. (C) Leaves of control (TRV:*GUS*), and *Sec5*- and *SGT1*-silenced *N. benthamiana* plants were infiltrated with *A. tumefaciens* strains harbouring constructs expressing *R1/AVR1*, *R3a/AVR3a* and *Rx/CP*. Cell death development was monitored and pictures were taken at 6 dpi. (D) Graphs showing the percentage of infiltration sites developing cell death mediated by co-agroinfiltration of *R1/AVR1, R3a/AVR3a* and *Rx/CP* at 6 dpi on leaves of control (TRV:*GUS*; ■) and *Sec5*- (■) and *SGT1*-silenced (□) *N. benthamiana* plants. Experiments were repeated three times and the graph shows the mean of three biological repeats. (N≥22).

Sec5-silenced plants show attenuated cell death responses

In order to quantify the level of cell death triggered by R1/AVR1 we analysed ion leakage as a measure for plant cell death. On the control plants, R1/AVR1-triggered responses could be observed clearly as early as 2 dpi. By 4 dpi the infiltration zone is dried-out and no longer suitable for measuring ion leakage. At 2 dpi relative ion leakage levels were already up and they continued to increase reaching 80% at 3 dpi, the latest time point measured (Figure 4A) and this coincided with the cell death responses visible on the infiltrated leaves.

To further confirm this we analysed the production of reactive oxygen species (ROS), another hallmark for cell death. Generation of superoxide anion (O_2^-) was detected by staining with nitro blue tetrazolium (NBT), and hydrogen

peroxide (H_2O_2) by staining with 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA). Upon GUS infiltration, that was used as a control, there is no green fluorescence in leaves from either control plants or *Sec5*- and *SGT1*-silenced plants which demonstrates no or little generation of H_2O_2 (Figure 4C). In contrast, infiltration with R1/AVR1 resulted in a strong fluorescence in control plants pointing to a large amount of H_2O_2 already at 1 dpi. In *SGT1*-silenced plants there is no H_2O_2 production at 2 dpi and this is in line with previous studies stating that silencing of *SGT1* abolishes proper functioning of NLR proteins. In *Sec5*-silenced plants the production of H_2O_2 at 3 dpi is clearly reduced. For O_2^- production detected with NBT staining we observed similar results (Figure 4D). These results obtained with ROS staining together with the ion leakage data demonstrate that cell death is attenuated in *Sec5*-silenced plants and lead to the conclusion that Sec5 is required for R1/AVR1-triggered HR.

Sec5-silencing enhances plant susceptibility and abolishes R1-mediated resistance

To study the role of Sec5 in defense against *P. infestans*, we performed infection assays on *Sec5*-silenced *N. benthamiana* plants with two *P. infestans* strains, 14-3-GFP and T20-2. Results obtained in detached leaf assays show that the lesions on *Sec5*-silenced plants are larger than the lesions on control plants (Figure 5), indicating that Sec5 is needed to counteract the pathogen.

To further investigate the potential role of Sec5 in R1-mediated resistance to *P. infestans*, we performed infection assays on *Sec5*-silenced *N. benthamiana* plants that express *R1*. Two days before inoculation, leaves of *Sec5*-silenced plants were agroinfiltrated with pGRAB-R1 on one half of the leaf and, as a control, pGRAB-GUS on the other half of the same leaf. Control and TRV:*SGT1* plants were treated in the same way. For inoculation we used a zoospore suspension of the *AVR1*-expressing *P. infestans* isolate T20-2 (van der Lee et al., 2001) and applied one droplet on each side of the leaf. The left half of leaves that express *GUS* developed lesions pointing to successful infection. In the presence of *R1* (right half), however, infection on control plants is blocked as expected, but is not blocked on *Sec5*-silenced and *SGT1*-silenced plants (Figure 6A). Quantification showed that in the absence of R1 more than 80% of the inoculated spots resulted in expanding lesions irrespective of the gene that was targeted by silencing (*Sec5*, *SGT1* or *GUS*) (Figure 6B). In the control plants (TRV:*GUS*) expression of R1

significantly reduced the percentage of successful infections to less than 20. Apparently, the transiently expressed R1 is able to recognize AVR1 resulting in an incompatible interaction. In contrast, in the *Sec5*- and *SGT1*-silenced plants half or more of the inoculations led to successful infection despite the presence of R1 (Figure 6B). These results indicate that similar to SGT1, Sec5 is required for optimal functioning of R1-mediated resistance.

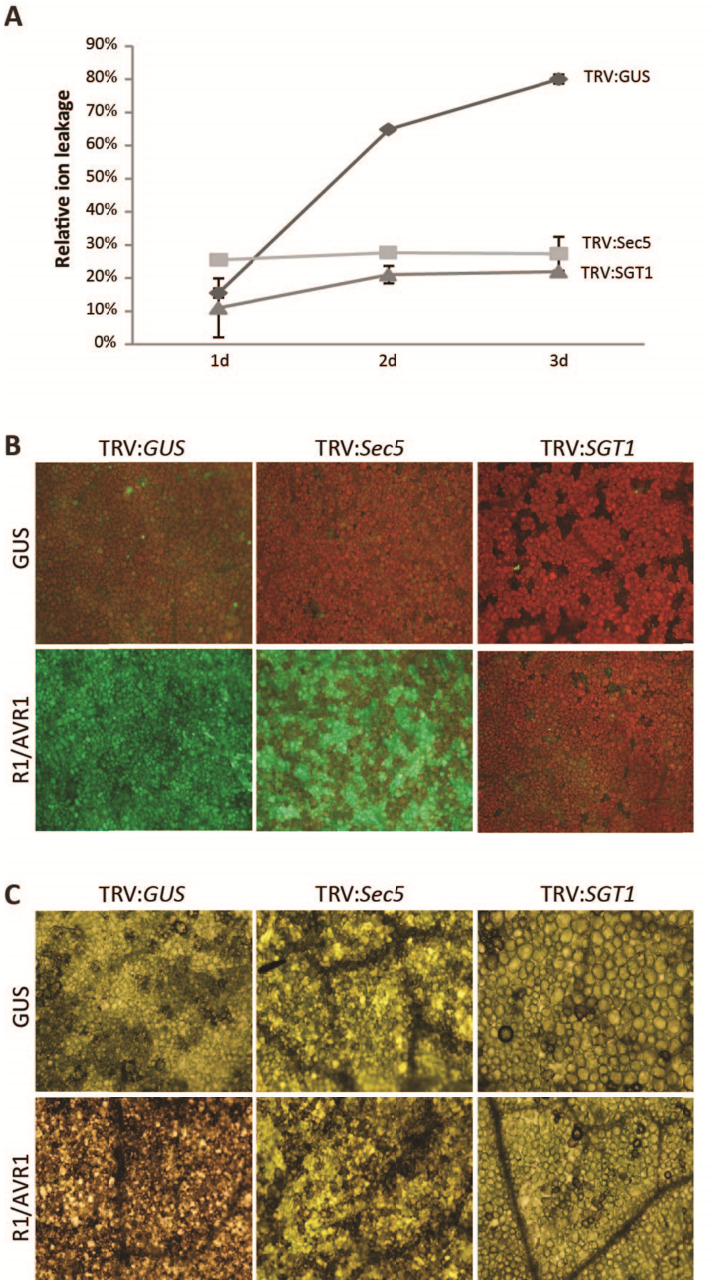

Figure 4. Cell death responses are attenuated in *Sec5*-silenced plants.

Quantification of cell death and visualization of ROS production induced by expression of R1/AVR1 in leaves of control (TRV:*GUS*), and *SGT1*- and *Sec5*-silenced *N. benthamiana* plants. In (A) ion leakage is measured at 1, 2 and 3 dpi with R1/AVR1. The Y-axes represent the relative conductivity in percentage. In (B) ROS production is visualized by staining H_2O_2 with DCFH-DA and in (C) by staining O_2^- with NBT. Samples were stained and photographed under the microscope at 1, 2, and 3 dpi. The pictures shown are taken from leaves at 1 dpi for TRV:*GUS* plants, 2 dpi for TRV:*SGT1* plants and 3 dpi for TRV:*Sec5* plants.

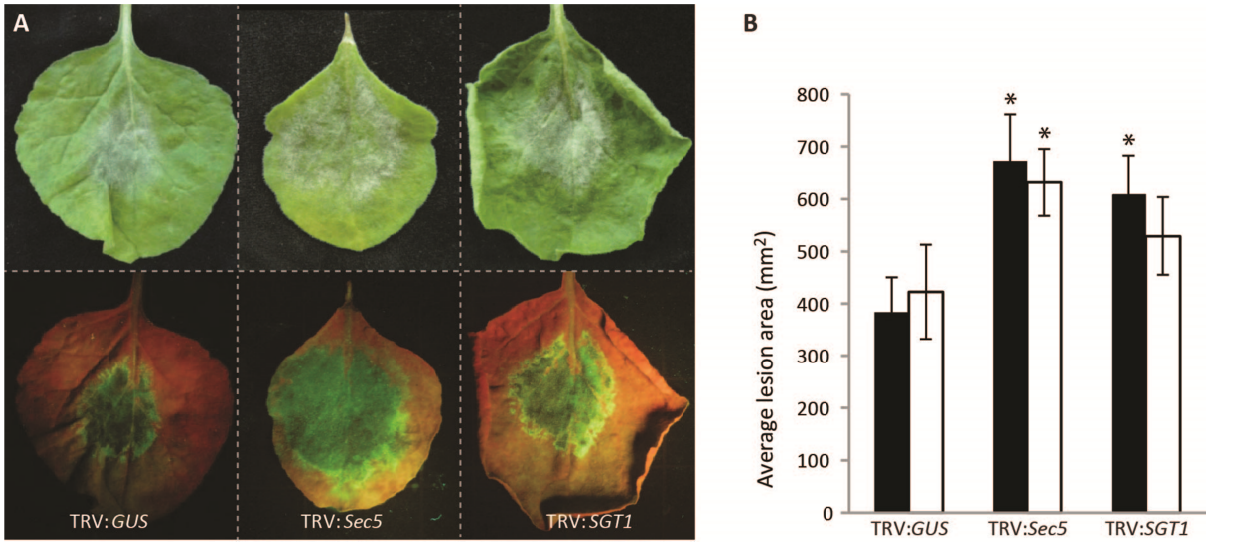

Figure 5. *Sec5*-silenced *N. benthamiana* plants show increased susceptibility to *P. infestans*.

Leaves of silenced plants were harvested three weeks after agroinfiltration with the silencing constructs TRV:*GUS*, TRV:*Sec5* or TRV:*SGT1* and drop-inoculated with *P. infestans* strains T20-2 and 14-3-GFP. (A) Pictures of detached leaves infected with strain 14-3-GFP were taken at 6 days after inoculation (dai), without (top) and with UV illumination. (B) Graph showing the lesion areas in mm^2 (Y-axis) at 6 dai with strains T20-2 (■) and 14-3-GFP (□). Error bar is from 8 replicates. The experiments were repeated three times with similar results. Significant differences are indicated with asterisks (N≥8, one sided Student's T-test, $P < 0.05$).

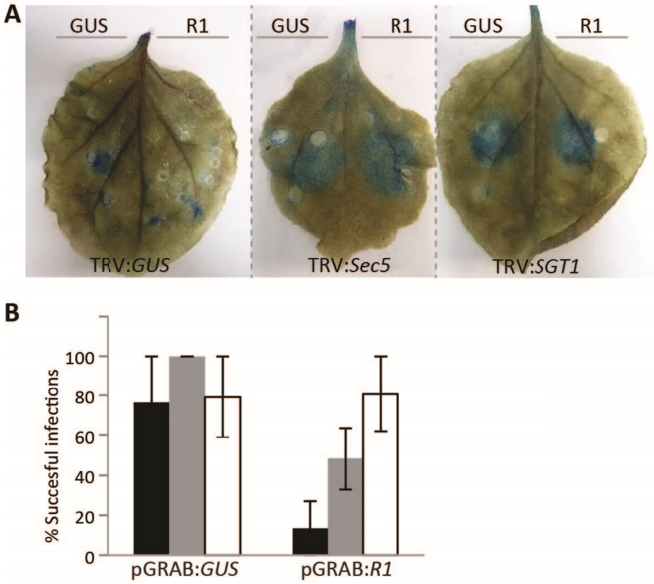

Figure 6. Sec5 is required for full R1/AVR1 HR.

(A) Lesions on leaves of *GUS*-, *Sec5*- and *SGT1*-silenced *N. benthamiana* plants that transiently express GUS (left) or R1 (right). At 2 days after agroinfiltration with PGRAB-GUS and PGRAB-R1 leaves were inoculated on each side of the leaf with a 10 µl drop containing 5000 zoospores from *P. infestans* strain T20-2. At 6 dai leaves were stained with trypan blue and photographed. (B) The percentage of total inoculated sites that show successful infection (Y-axis). The means of three biological replicates are shown (■, TRV:*GUS*; ■, TRV:*Sec5*; □,TRV:*SGT1*).

Sec5-silencing and *AVR1* expression impair callose deposition

The increased susceptibility due to *Sec5*-silencing might suggest that Sec5 is required for PTI. To test this we used callose deposition as a marker for PTI. We analysed the level of callose deposition in *Sec5*-silenced *N. benthamiana* plants infiltrated with the *hrcC* mutant of the *Pseudomonas syringae* strain DC3000 (*Pst ΔhrcC*). This mutant can no longer secrete effectors and supress callose deposition. Microscopic analysis revealed that control plants displayed a significant level of callose deposition at 14 hours after infiltration with *Pst ΔhrcC.* In contrast, callose deposition was not observed in *Sec5* and *SGT1*-silenced plants (Figure 7A). Hence, it can be concluded that *Sec5* is required for callose deposition in *N. benthamiana* indicating a role for Sec5 in PTI.

The finding that AVR1 interacts with Sec5 (Figure 1 and 2B) raised the question to what extent expression of AVR1 can mimic the effect of *Sec5*-silencing on PTI and callose deposition. We therefore analysed callose deposition in *N. benthamiana* plants that transiently express Avr1. Two days after agroinfiltration with AVR1-myc, AVR1-like-myc, or GUS-myc constructs, leaves were infiltrated with *Pst ΔhrcC* to induce callose deposition. Microscopic analysis revealed that at 14 hours after infiltration the control leaves expressing GUS-myc displayed significant levels of callose deposition as expected. The presence of AVR1, however, impaired the callose deposition. Hardly any callose was observed and this was strikingly different with the situation in leaves expressing AVR1-like (Figure 7B). This difference is in line with the finding that AVR1, but not AVR1-like can manipulate Sec5 function.

Sec5 is required for secretion of PR-1 to the apoplast

The exocyst of which Sec5 is a subunit, is known to function in vesicle trafficking. In plants the exocyst might play a role in secretion of antimicrobial compounds including Pathogenesis-Related (PR) proteins that are often induced upon pathogen attack. PR-1 is one of the PRs that is secreted to the apoplast and is frequently used as marker for plant immune responses (Kalde et al., 2007; Wang et al., 2005; Wick et al., 2003). Here we used PR-1 as marker to monitor the involvement of Sec5 in secretion. We exposed *Sec5*-silenced *N. benthamiana* plants (TRV:*Sec5*) and control plants to salicylic acid (SA) to induce PR-1 accumulation and subsequently analysed PR-1 levels in the apoplast. Apoplastic fluid (AF) was collected and proteins were separated by SDS-PAGE. Western blot

analysis using an antibody that is specific for PR-1 and detects a single band at the size of 14 kDa, and this revealed that there was less PR-1 in AF collected from *Sec5*-silenced plants than in AF collected from control plants (Figure 8), whereas in the total protein extracts there was no difference. This result indicates that Sec5 contributes to PR-1 secretion to the apoplast, and indeed points to the malfunctioning of exocytosis in *Sec5*-silenced plants.

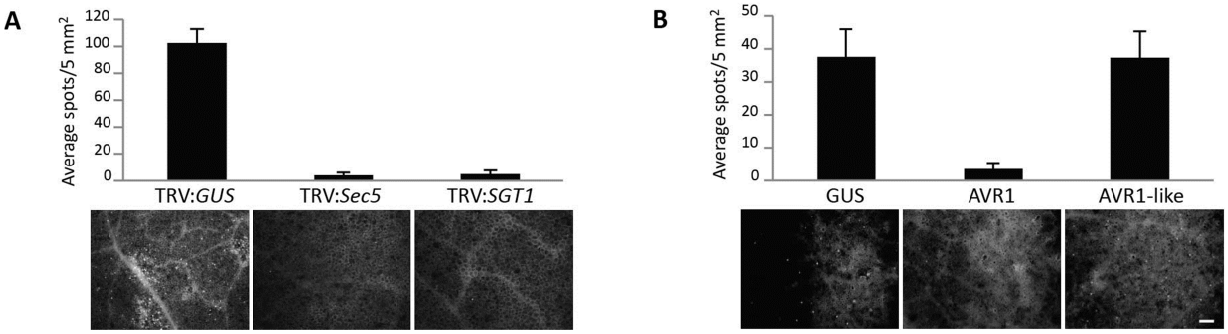

Figure 7. *Sec5*-silencing and AVR1 expression impair callose deposition.
(A) Leaves from *GUS*-, *Sec5*- and *SGT1*- silenced *N. benthamiana* plants were infiltrated with *Pst* DC3000 *ΔhrcC* ($1.0x10^8$ cfu/mL) and 14 hours later, stained with aniline blue. (B) *N. benthamiana* leaves expressing GUS-myc, AVR1-myc or AVR1-like-myc in combination with P19 were infiltrated with *Pst* DC3000 *ΔhrcC* mutant and after 14 hours stained with aniline blue. Callose deposition was quantified by counting the average number of spots per microscopy picture and the Y axes of the graph shows the average number of spots per 5 mm^2 (N=9). Representative pictures are shown below the graph. Bar=200 µm.

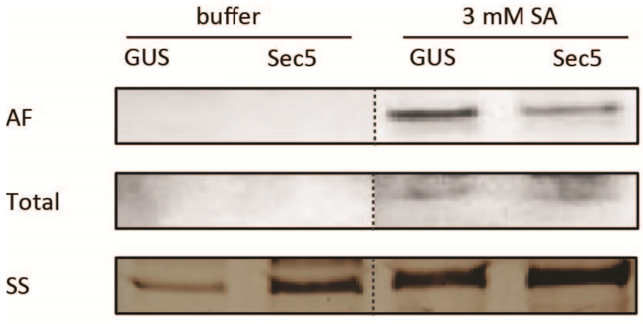

Figure 8. Sec5 is required for PR-1 accumulation to the apoplast.
Immune blots probed with PR-1 antibody and containing apoplastic fluid (AF) and total protein extract (total) from leaves of control (GUS) and *Sec5*-silenced (Sec5) *N. benthamiana* plants treated with buffer or 3 mM salicylic acid. The lower panel shows a section of the silver stained (ss) gel containing the apoplastic fluid proteins.

Discussion

The success of a plant pathogen is determined by its ability to overcome the intrinsic defense barriers that safeguard the plant from being attacked. In the battle between plant and pathogen, effectors play a major role. Biotrophic pathogens secrete a large variety of effectors, many of which are believed to be translocated into the host cell where they disturb the host machinery at multiple levels. In this study we provide evidence that the oomycete pathogen *P. infestans* exploits effectors to hijack exocytosis, a cellular process that is not only essential for normal plant development but also for plant defense (Hala et al., 2008; Wu et al., 2013; Pecenkova et al., 2011). During exocytosis secretory vesicles are directed to, and fused with the plasma membrane and this site-directed or focal secretion is important, for example for the formation of papillae to block penetration by the pathogen, or for delivery of anti-microbial compounds to infection sites to inhibit the pathogen. Indications that plant pathogens interfere with defense-related focal secretion arose from studies on fungal and bacterial pathosystems (Nomura et al., 2006; Ham et al., 2006; Driouich et al., 1997; Consonni et al., 2006) and on Avrblb2, a *P. infestans* RXLR effector that prevents secretion of the papain-like cysteine protease C14 into the apoplast (Bozkurt et al., 2011). The latter is one of the few studies so far that have pinpointed host targets for RXLR effectors and, apart from C14, none of the known RXLR host targets has a direct role in the secretion machinery or is secreted (Bos et al., 2010; Bozkurt et al., 2011; Bouwmeester et al., 2011; McLellan et al., 2013). Here we focus on AVR1, an RXLR effector of *P. infestans* that elicits a resistance response in potato plants carrying a cognate *R* gene, in this case *R1*, but subverts host defense in the absence of *R1.* To understand how AVR1 manipulates the host and activates R1 mediated resistance, we searched for host targets in a Y2H screening and identified a candidate that has a function in exocytosis. Based on our findings we hypothesize that AVR1 indirectly abolishes PR-1 secretion and callose deposition by manipulating or suppressing the function of Sec5, one of the subunits of the exocyst complex. Our findings are discussed below, and incorporated in a model. To our knowledge this is the first report describing an exocyst component as a direct target of an oomycete RXLR effector.

Upon pathogen attack, the basic cell machinery is alerted and the cell starts to secrete cell wall appositions and callose to the infection sites. That this type of focal secretion requires adequate functioning of the vesicle trafficking machinery

is demonstrated by the fact that mutants lacking the SNARE protein SYP121 (*pen1*) show defects in papillae formation. In *pen1* mutants papillae formation is delayed resulting in compromised penetration resistance to the powdery mildew *Blumeria graminis* f. sp. *hordei* (Assaad et al., 2004; Kwon et al., 2008). Enzymes involved in the synthesis of callose, one of the structural components of papillae, are transported via vesicles towards the plasma membrane (Nielsen et al., 2012; Xu and Mendgen, 1994) and, as shown by Pecenkova et al. (2011), perturbation of the exocyst complex can also have an impact on papillae formation. When they challenged Arabidopsis mutants that lack the exocyst subunit exo70B2 with *Blumeria graminis* f. sp. *hordei* they observed abnormal papillae with vesicular halos. Moreover, *exo70B2* as well *exo70H1* mutants showed increased susceptibility to *P. syringae* pv *maculicola.* In line with this we observed that depletion of the exocyst component Sec5 in *N. benthamiana* abolishes callose deposition (Figure 7A and 7B) and increases the susceptibility to *P. infestans* (Figure 5). This demonstrates that Sec5 is directly or indirectly involved in transporting callose biosynthetic enzymes to the plasma membrane and is needed for optimal defense against *P. infestans*. The fact that callose deposition was found to be suppressed by AVR1 (Figure 7C and 7D) is consistent with the finding that AVR1 sequesters Sec5 (Figure 1). Apparently, AVR1 manipulates focal secretion by interacting with the Sec5 protein.

A role for Sec5 and the exocyst in focal secretion was further demonstrated by our finding that silencing of *Sec5* in *N. benthamiana* impaired the secretion of the pathogenesis-related protein PR-1 to the apoplast (Figure 8A). Pathogenesis-related proteins (PRs) are well known as major constituents of the antimicrobial cocktails that plants produce in response to pathogen attack. They usually have the typical N-terminal signal peptide for secretion and mainly accumulate in the apoplast (Joosten et al., 1990; Van Loon and Van Strien, 1999). A previous study already indicated that the vesicle trafficking machinery downstream of the exocyst is important for focal secretion of PR-1. Depletion of the plasma membrane specific syntaxin SYP132 in *N. benthamiana* resulted in delayed PR-1 secretion and compromised both Pto-mediated resistance and basal defense to *P. syringae* pathovar *tomato* (Kalde et al., 2007). In addition, expression of the pathogen-induced gene *AtSNAP33* which encodes a t-SNARE that is involved in vesicle associated secretion and forms a complex with the PEN1 syntaxin and VAMP721/722 (Yun et al., 2013), was found to be correlated with PR-1

accumulation into the apoplast (Wick et al., 2003). As yet we have no evidence that the presence of AVR1 affects PR-1 secretion. Since AVR1 targets Sec5 whereas AVR1-like does not, we had anticipated to find differences in PR-1 levels in AF of leaves expressing AVR-1 and AVR1-like. However, no differences were found not even with the empty vector control. For this assay we used Agrobacterium-mediated transient transformation to express AVR1, AVR1-like or the empty vector control. We suspect that the damage caused by the agroinfiltration already resulted in defense responses including PR-1 secretion making it impossible to discriminate potential differences 4 days after spraying SA.

Studies in mammalian systems have demonstrated a role for Sec5 that seems to be independent from its role as an exocyst component. Sec5 was found to participate in Toll-like receptor mediated innate immune signaling by interacting with and activating a TANK binding Kinase 1 (TBK1) (Simicek et al., 2013; Chien et al., 2006; Mantovani and Balkwill, 2006; Ishikawa et al., 2009; Ishikawa and Barber, 2008). Our data suggest a similar dual role for Sec5 in plants. We observed that Sec5 is required for resistance mediated by the NLR R1. In parallel, we observed that Sec5 is required for an efficient HR elicited by co-expressing R1 with AVR1, and this is consistent with the hypothesis that the higher number of lesions in *Sec5*-silenced leaves that transiently express *R1* is due to interference of AVR1-R1 HR. This attenuated HR was not observed with other AVR-NLR pairs, including the NLR R3a which, similar to R1, recognizes a *P. infestans* RXLR effector and confers resistance to potato late blight (Figure 3F). Apparently, Sec5 is involved in some but not all immune signalling pathways that lead to ETI. Such specificity was also observed for the putative phosphatase BSL1 which is required for late blight resistance mediated by the NLR R2. AVR2 but not AVR2-like, was able to promote the interaction of BSL1 with R2 to initiate R2-mediated resistance and HR (Saunders et al., 2012). Similar to Sec5, BSL1 did not affect the recognition of R3-AVR3a and Rx-CP. Proper functioning of the virus resistance protein Rx relies on physical interaction with a host Ran GTPase-activating protein 2 (RanGAP2), which is essential for maintaining the nucleocytoplasmic partitioning of Rx (Tameling et al., 2010). Whether or not BSL1 or RanGAP2 play a role in AVR1-R1 recognition is not known.

P. infestans has the capacity to produce hundreds of RXLR effectors. The composition of the cocktail of RXLR effectors varies among isolates. Some effectors are not crucial; deletions or frame shift mutations do not affect viability

or pathogenicity but help the pathogen to circumvent recognition by a host carrying a cognate *R* gene. Examples of such redundant effectors are *P. infestans* Avr4 (van Poppel et al., 2008) and *P. sojae* Avr1d (Yin et al., 2013). Their loss is likely compensated for by other effectors in the cocktail while others seem to be indispensable. For example, every *P. infestans* isolate has AVR3a and AVRblb1. In AVR3a the variation in the pathogen population is limited to two amino acids ($AVR3a^{KI}$ or $AVR3a^{EM}$) which are the determinants for recognition by *R3a* plants (Armstrong et al., 2005). In AVRblb1 (IPI-O) there is more variation with multiple genes in one isolate and it is the combination of IPI-O variants that determines compatibility or incompatibility with *Rpiblb1* plants (Champouret et al., 2009). In this respect AVR1 resembles AVR3a and AVRblb1; every isolate analysed so far has either AVR1 and is recognized by *R1* plants, or it has AVR1-like and is compatible with *R1* plants. However, AVR1-like does not sequester Sec5, so isolates lacking AVR1 might have other means to target vesicle trafficking for facilitating *P. infestans* colonization. Indications that there are other RXLR effectors apart from AVR1 that target the vesicle trafficking machinery to hamper host focal secretion come from Bozkurt et al. (2011) who showed that the RXLR effector Avrblb2 modulates focal secretion of C14 and from Bos et al. (2010) who identified Sec3 as a candidate host target of AVR3a by Y2H.

In our model we hypothesize that upon infection the plant cell activates its defense machinery by secreting PR-1 proteins into the apoplast and depositing callose at the infection site where the pathogen is. To break this defense *P. infestans* exploits AVR1 which has the capacity to stabilize Sec5 and in this way blocks focal secretion of PR proteins and callose to the infection site (Figure 9A). When infecting plants that carry the NLR R1, *P. infestans* is not aware of the fact that R1 is present and also exploits AVR1 to sequester Sec5 (Figure 9B). In the absence of Sec5 HR and R1-mediated resistance is attenuated demonstrating that Sec5 is an important component in the signalling pathways that culminate in the HR. Unlike AVR1, AVR1-like cannot sequester Sec5. Nevertheless, *P. infestans* strains that have AVR1-like instead of AVR1 can still cause infection irrespective of presence or absence of R1. AVR1-like does not prevent deposition of callose, so focal secretion may still function (Figure 9C). Since we do not yet know the host target of AVR1-like we can only speculate; either AVR1-like breaks the defense at another level overruling callose deposition or there are other effectors that target exocytosis and block focal secretion (Figure 9D).

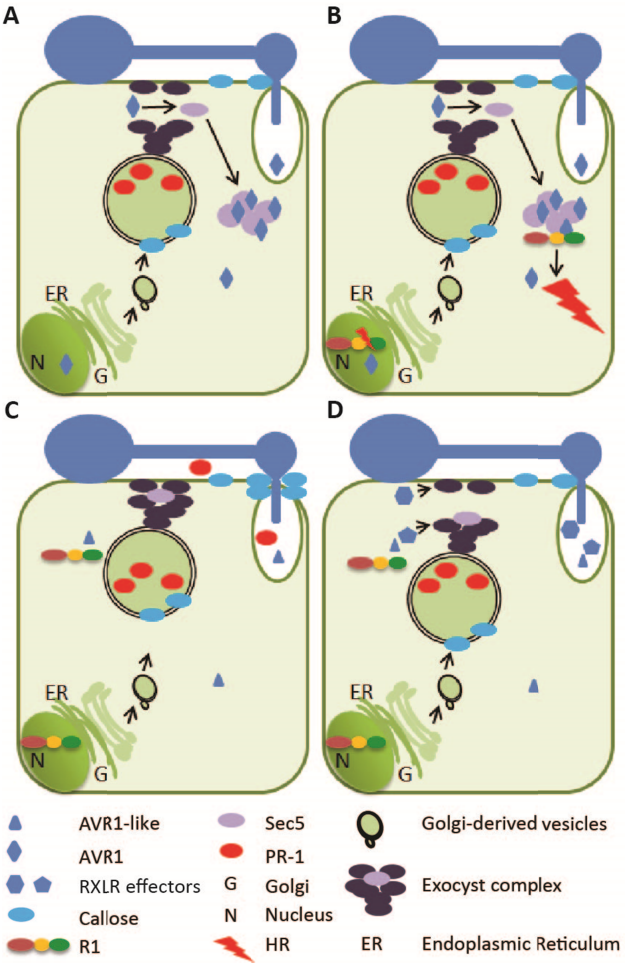

Figure 9. *P. infestans* RXLR effector AVR1 and its host target Sec5.
Models depicting the interaction between AVR1 and Sec5 in plant cells colonized by *P. infestans*. Germ tubes emerging from cysts form an appressorium. After penetration a haustorium is formed from which RXLR effectors are translocated into the host cell. (A) In response to pathogen invasion, the host cell secretes antimicrobial compounds (including the pathogenesis related protein PR-1) and deposits callose at the site of penetration to hamper infection (as depicted in C). However, when AVR1 is secreted by *P. infestans* and is translocated from the haustorium into the host cell, it targets and stabilizes Sec5 in the cytoplasm. The exocyst complex is thus incomplete and not able to mediate focal secretion of PR-1 and callose. (B) In *R1* containing plants, the resistance protein R1 recognizes AVR1 resulting in the activation of a Sec5-dependent HR. Recognition of AVR1 is either directly or via a complex containing Sec5 sequestered by AVR1. (C) and (D) When *P. infestans* secretes AVR1-like instead of AVR1 there is no HR in R1 containing plants and no sequestering of Sec5 by AVR1. Since colonization is not blocked, *P. infestans* can either withstand the inhibitory activity of PR-1 and overcome the barrier caused by callose deposition (C) or, more likely, it secretes other RXLR effectors that target the exocyst and/or vesicle trafficking machinery to hamper the focal secretion (D).

Materials and methods

Plant material and pathogen inoculation

N. benthamiana and tomato (cv. Moneymaker) were grown in a greenhouse under standardized conditions. *P. infestans* isolates 14-3-GFP and T20-2 were maintained on rye sucrose agar medium at 18°C. *P. infestans* zoospores were isolated and detached leaf assays were performed as previously described (Champouret et al., 2009). In short, *N. benthamiana* was inoculated on the abaxial leaf surface with a 10 µl droplet of a suspension with $5x10^5$ zoospores/mL, and incubated at high humidity at 18°C, with the first 24 hours in the dark. Lesion areas were determined at 6 days after inoculation (dai) as described by Vleeshouwers et al. (1999). *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 *ΔhrcC* was grown at 28°C in King's B medium and resuspended to OD600 of 0.5 (approximately $1.0x10^8$ cfu/mL) in 10 mM $MgCl_2$ before infiltration into *N. benthamiana* leaves (Kim et al., 2011).

Plasmid construction

A 578 bp fragment of *Sec5* was PCR amplified from *N. benthamiana* cDNA using primers Sec5-vigs-F and Sec5-vigs-R and cloned into the binary vector pTRV2 (Velasquez et al., 2009) using the restriction sites *Bam*HI and *Sac*I to generate TRV:*Sec5* (Figure S1; Supplemental Table 1). Potato *Sec5* (*StSec5*) was amplified using primers Sec5F and Sec5Rwostop (Figure S1; Supplemental Table 1) from potato cDNA. Subsequently, fragments were cloned into pENTR/D-TOPO entry vector (Invitrogen) and recombined into vector pSOL2094 resulting in pSOL-StSec5 (GFP-StSec5).

P. infestans effector genes *AVR1* (PITG_16663.2), *AVR1-like* (PITG_06432.1), *ipiO1 (*PITG_21388.2; GenBank L23939.1*)* and *ipiO4* (GenBank GQ371191.1) were amplified without signal peptides using Gateway-compatible primers (Supplemental Table 1; Figure S1), and cloned into pENTR/D-TOPO entry vector (Invitrogen). LR reactions involving various destination vectors resulted in pGWB20-*AVR1*-10myc (AVR1-myc), pGWB20-*AVR1-like*-10myc (AVR1-like-myc), and pGWB20-10myc (empty vector). To generate Y2H prey plasmids, pENTR vectors carrying the effector genes were recombined into pDEST32 which contains the GAL4 DNA binding domain (GAL4 DNA-DB) resulting into pDEST32-*AVR1*, pDEST32-*AVR1-like*, pDEST32-*ipiO1* and pDEST32-*ipiO4*.

AVR1:YC and AVR1-like:YC were generated by recombining pENTR/D-TOPO entry clones containing AVR1 or AVR1-like with pGREENII-YFPC (Zhong et al., 2008), and YN:StSec5 was generated by recombining StSec5 containing pENTR/D-TOPO entry clone with PCL112 (Bos et al., 2010) using Gateway LR recombination.

Coding sequences of *CRN2*, *AVR1* and *R1* were amplified using appropriate primers (Supplemental Table 1), and cloned into the binary vector pGRAB using *Not*I and *Asc*I restriction sites for *AVR1* and *Eco*R1 and *Not*1 for *R1* and *CRN2*. Plasmids for transient expression assays were transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation.

Virus-induced gene silencing

Virus-induced gene silencing (VIGS) was performed as previously described (Peart et al., 2002). Constructs TRV:*GUS* and TRV:*SGT1* were used as controls (Tameling and Baulcombe, 2007). Two weeks old *N. benthamiana* and tomato plants were infiltrated with *Agrobacterium* suspensions containing pTRV2 derivatives and pTRV1 in a 1:1 ratio (final OD600 of 1.0 for *N. benthamiana* and 1.6 for tomato). Three to four weeks later, the fifth and sixth leaf above the infiltrated leaf of *N. benthamiana*, and the fourth and fifth leaf of tomato plants were used for further analysis.

Agroinfiltration assays

A. tumefaciens AGL1 strains carrying binary vectors were cultured as described (Champouret et al., 2009; Van der Hoorn et al., 2000). For co-expression we used a 1:1 ratio for all combinations. The final OD for cell death inducers was adjusted according to their strength of the HR on *N. benthamiana*. For R1/AVR1 the final OD was 0.3 and for other elicitors and R/AVR 0.5. Agroinfiltrated plants were kept in a 25°C climate chamber with 10 hours photoperiod and 70% relative humidity (RH).

For co-IP experiments, leaves of *N. benthamiana* were co-agroinfiltrated with GFP-StSec5 combined with either AVR1-myc, AVR1-like-myc or empty vector, and P19 (in a 1:1:1 ratio) with a final OD of 0.3. For BiFC, agrobacterium strains that harbouring complementary halves of YFP were agroinfiltrated into *N. benthamiana* leaves together with P19 in a 1:1:1 ratio with a final OD of about 0.3.

Salicylic acid treatments of *N. benthamiana* plants and apoplastic fluid isolation

N. benthamiana plants were sprayed with 3 mM SA in 20 mM sodium phosphate buffer (pH 6.5) containing 0.02% Silwet L-77 for four consecutive days (El Oirdi et al., 2011). Control treatments were performed with buffer containing 0.02% Silwet L-77 without SA. Apoplastic fluid (AF) was collected at the fourth day as described by Joosten (2012).

Quantitative RT-PCR

Total RNA was isolated by NucleoSpin RNA plant mini-kit (Clontech). RNA concentration was quantified and 1 µg used as template for reverse transcription into cDNA with M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instruction. The cDNA was diluted 10 times and 2 µl was used per PCR reaction with SYBR Green master mix (Promega) and gene-specific primers (Table S1; Figure S1). Q-RT-PCR was performed in triplicate on a Bio-Rad 7300 thermocycler, and gene expression was quantified and normalized to actin expression.

Yeast-two hybrid assay

Yeast screening was conducted using the ProQuest Two-Hybrid System (Invitrogen) with pDEST32:*AVR1* as bait against a potato Y2H library as described by Bos et al. (2010). Approximately 250 His-positive yeast colonies were randomly selected in the first screening and plasmids were isolated for sequencing. Three *StSec5* clones that were picked up in the initial Y2H screen, i.e. *StSec5-a* (pDEST22:*Sec5* $^{3\text{-}1100\ AA}$), *StSec5-b* (pDEST22:*Sec5* $^{99\text{-}1100\ AA}$) or *StSec5-c* (pDEST22:*Sec5* $^{157\text{-}1100\ AA}$) were co-transformed with pDEST32:*AVR1*, pDEST32:*AVR1-like*, pDEST32:*ipiO1* or pDEST32:*ipiO4.* To check their interaction strength, yeast co-transformants were first selected on SD agar plates lacking the amino acids Trp and Leu (SD-W-L). Subsequently, co-transformants were selected on His-deficient SD plates (SD-W-L-H) supplemented with 25 mM 3-Amino-1,2,4-triazole (3-AT) and Ura-deficient SD plates (SD-W-L-U). Yeast controls showing strong, intermediate and no interaction were provided by the ProQuest Two-Hybrid System (Invitrogen).

Staining and microscopy

Accumulation of O_2^- and H_2O_2 were visualized by nitro blue tetrazolium (NBT) and 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) staining, respectively. *N. benthamiana* leaves were vacuum infiltrated with staining solutions and incubated 2 hours for NBT staining (2 mM NBT in 0.1 mM Tris-HCl buffer), and 0.5-1 hour for DCFH-DA staining (1% DCFH-DA in DMSO, diluted 1:100 in PBS, pH 7.4) (Rossetti and Bonatti, 2001). Stained tissues were analysed by brightfield and fluorescence microscopy using a GFP filter to detect O_2^- and H_2O_2, respectively (Nikon 90i epifluorescence microscope, Nikon, Amstelveen, The Netherlands). Callose deposition was analysed at 14 hours after *Pst* DC3000 *ΔhrcC* infiltration. Leaf samples were stored with ethanol to remove chlorophyll and then stained with 0.25% (w/v) aniline blue in 150 mM K_2HPO_4 (pH 9.5) for 1 hour (Bouwmeester et al., 2011). Callose was visualized using epifluorescence microscopy (DAPI filter, EX340-380, DM400, BA 435-4850). Three pictures were taken randomly from each leaf sample and experiments were repeated three times with three replicates each time. For bimolecular fluorescence complementation, a Zeiss LSM 510-Meta confocal microscope was used. The excitation wavelengths used for YFP and GFP are 514 nm and 488 nm respectively. The microscopy pictures were taken with identical settings.

Co-immunoprecipitation and immunoblot analysis

N. benthamiana leaves were harvested 3 days post agroinfiltration (dpi) and grinded in liquid nitrogen. Proteins were extracted using RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with 5 mM DTT and one complete proteinase inhibitor tablet (Roche) per 50 mL extraction buffer. For co-immunoprecipitation (Co-IP) 5 mL total protein extract was mixed with 20 µl GFP-trap_A® beads (Chromotek) and incubated at 4°C for 1.5 hours. Protein complexes attached to the beads were washed and collected, and then boiled for 5 min with loading buffer (300 mM Tris-HCl pH 6.8, 8.7% SDS, 5% β-mercaptoethanol, 30% glycerol and 0.12 mg/mL bromophenol blue) and separated on a SDS-PAGE gel followed by transfer to an Immune-Blot® PVDF membrane (Bio-Rad). For detection of myc fused proteins the monoclonal antibodies α-myc (Sigma-Aldrich, 9E10) were used at 1:2000 dilutions and followed by incubation with a second antibody anti-mouse Ig-HRP (Amersham). For detection of GFP fused proteins the α-GFP (Anti-GFP-HRP, 130-091-833, MACS

antibodies) were used at 1:2000 dilutions. Protein bands were detected using SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific).

PR-1 was detected using PR-1 antibody as described by Joosten et al. (1990). Antigen-antibody complexes were detected using 1:2000 diluted Goat anti-Rabbit-HRP (Anti-Rabbit IgG-HRP, A9169, Sigma).

Ion leakage measurements

From agroinfiltrated *N. benthamiana* leaves six leaf discs (8 mm in diameter) were punched out from each infiltrated area and incubated in 4 mL distilled water at room temperature for 2 hours with slow shaking. After incubation, samples of the solution were taken for measuring sample conductivity. The leaf discs were kept in the solution and boiled for 15 minutes. After boiling the solution was sampled again for the total conductivity. Relative ion leakage is represented by the percentage of the sample conductivity divided by the total conductivity (Kim et al., 2003). Experiments were repeated three times with three replicates of each sample.

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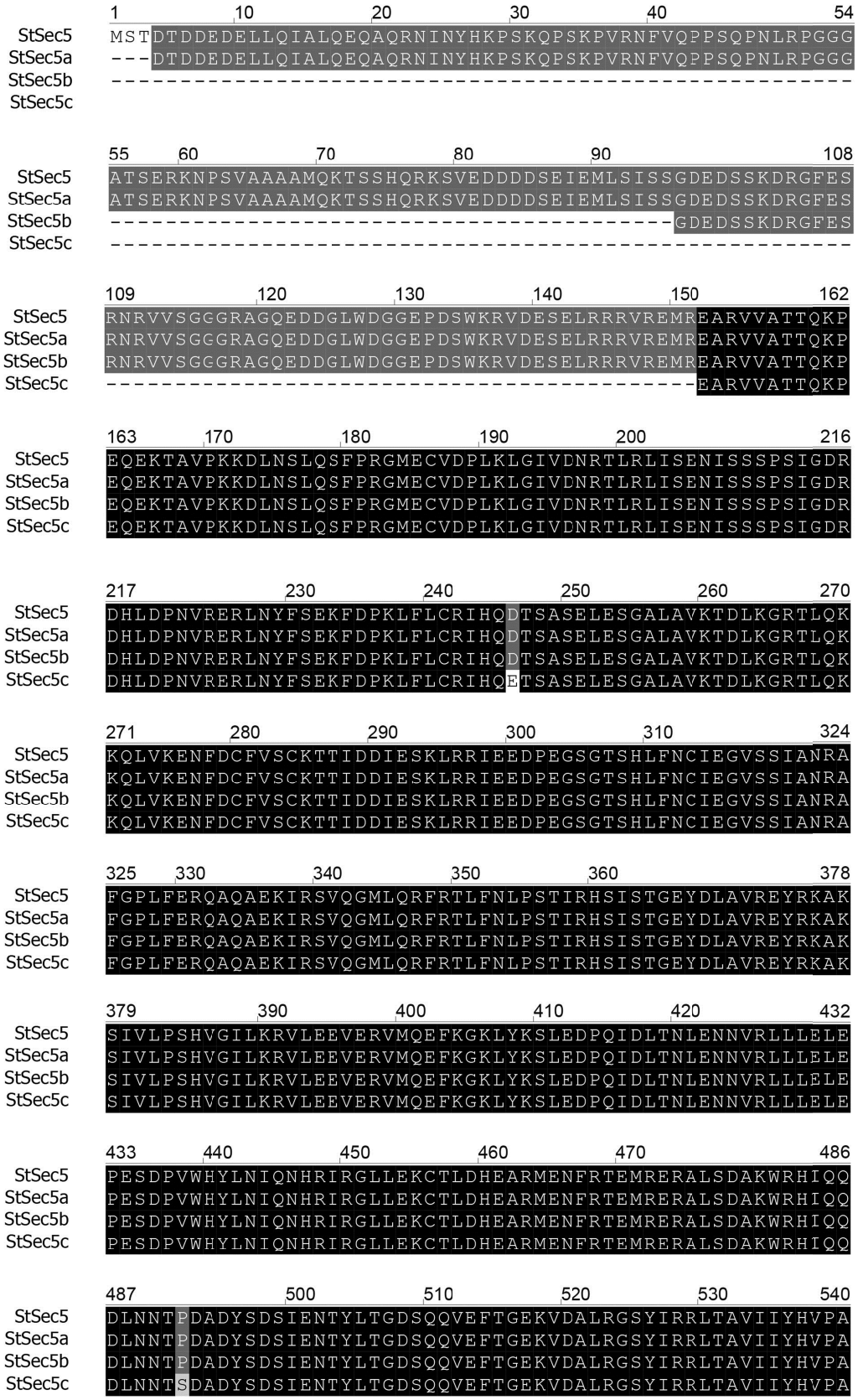
Supplementary information



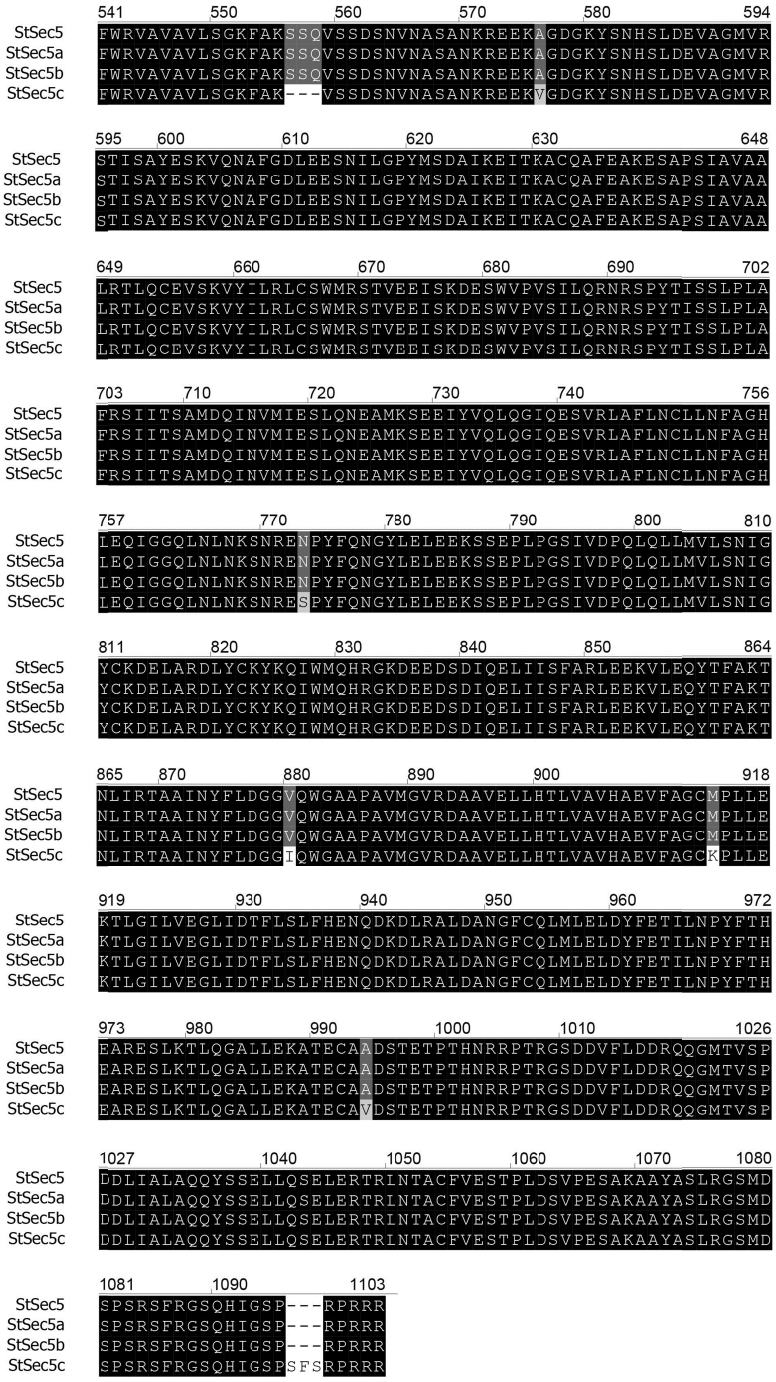


Figure S1. Prediction of the StSec5 protein sequence based on alignment with deduced amino acid sequences of three cDNA clones selected in a Y2H screen with AVR1 as bait. Clone StSec5-a is predicted to be almost full length based on comparison with a cDNA sequence from *Solanum lycopersicum* (Solyc04g071350) with three amino acids missing at the N-terminus. The overlapping part of clone StSec5-a and StSec5-b are completely conserved while clone StSec5-c has a few SNPs. It is not known if the sequence variants represent two copies in the potato genome or variation between cultivars. The latter is possible because the prey cDNA library was composed of a mixture of two cultivars. We used the StSec5 protein sequence shown in this figure as the sequence representing the potato Sec5 protein.

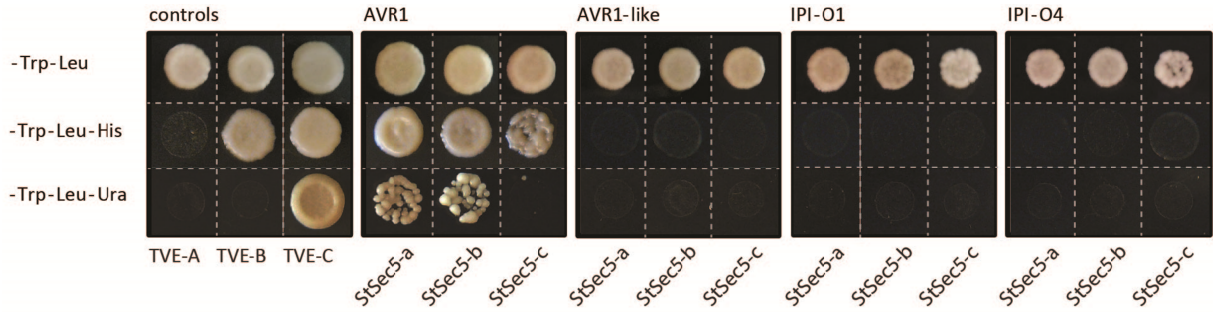


Figure S2. Y2H analysis illustrating that AVR1 specifically interacts with Sec5.
Yeast was co-transformed with a Y2H prey vector containing the three positive clones of Sec5 (a, b and c) from the first screening with bait vector containing AVR1, AVR1-like, IPI-O1 and IPIO-4. TVE-A, B and C are controls included in the Invitrogen Y2H kit that show no interaction (only growth on selection medium lacking Trp and Leu [–Trp-Leu]), intermediate interaction (able to grow on medium lacking Trp, Leu and His [–Trp-Leu-His]) and strong interaction (able to grow on medium lacking Trp, Leu and Ura [–Trp-Leu-Ura]).

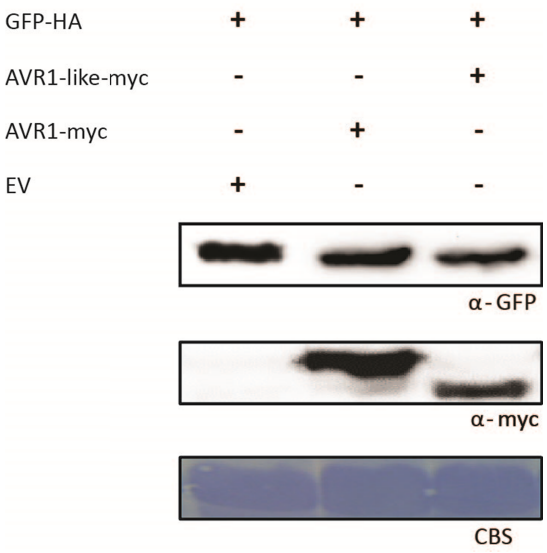


Figure S3. AVR1 does not stabilize GFP-HA.
Co-expression with AVR1 does not affect the steady state levels of GFP-HA. Total protein extracts from *N. benthamiana* leaves that were co-agroinfiltrated with GFP-HA combined with empty vector (EV), AVR1-myc or AVR1-like-myc at 3 dpi were separated on gel and blotted. GFP- and myc-tagged fusion proteins were detected by probing the western blots with α-GFP and α-myc. The result is representative for three independent experiments. CBS: Coomassie Blue staining of the blot containing total protein extracts showed equal loading in each lane based on the 50 kDa RuBisCo band.

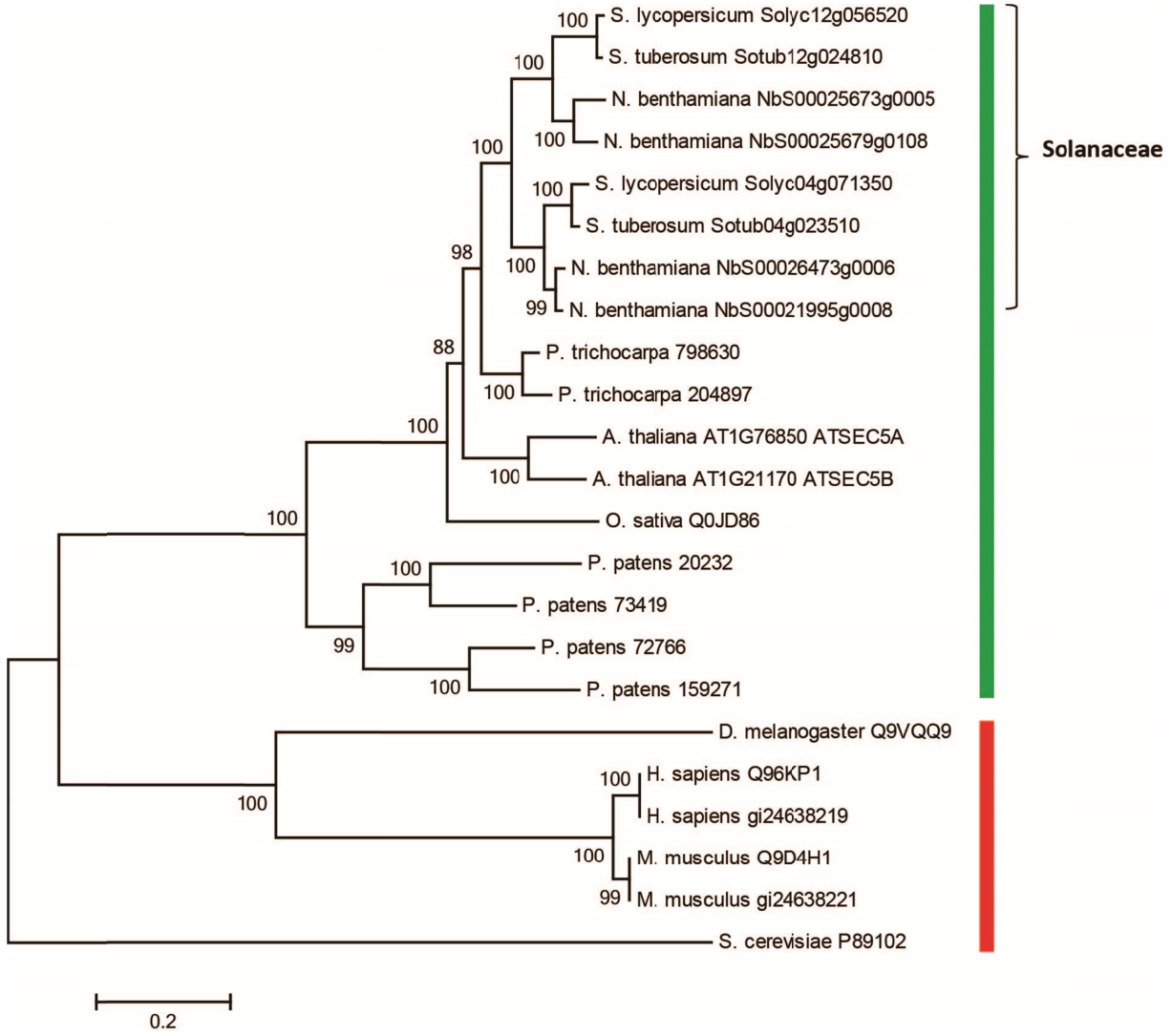
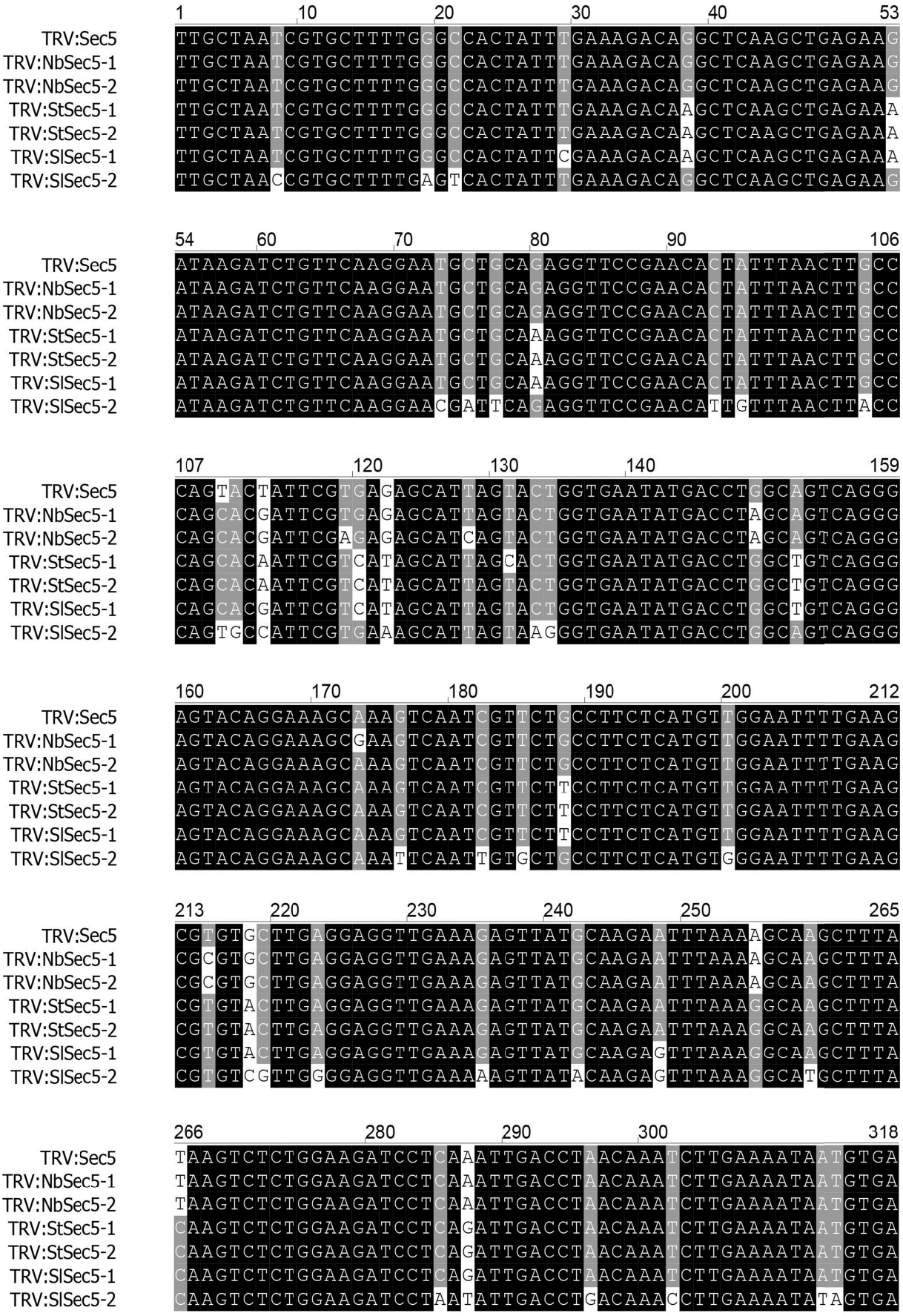


Figure S4. Phylogenetic tree of predicted Sec5 proteins from plants, animals and yeast. The tree was generated using MEGA4, Neighbour Joining with bootstrap of 10000. The predicted Sec5 proteins in Solanaceous plants are highly conserved and cluster together.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TRV:StSec5-1 TTGCTAATCGTGCTTTTGGGCCACTATTTGAAAGACAAGCTCAAGCTGAGAAA
TRV:StSec5-2 TTGCTAATCGTGCTTTTGGGCCACTATTTGAAAGACAAGCTCAAGCTGAGAAA
TRV:SlSec5-1 TTGCTAATCGTGCTTTTGGGCCACTATTCGAAAGACAAGCTCAAGCTGAGAAA
TRV:SlSec5-2 TTGCTAACCGTGCTTTTGAGTCACTATTTGAAAGACAGGCTCAAGCTGAGAAG
54 60 70 80 90 106
TRV:Sec5 ATAAGATCTGTTCAAGGAATGCTGCAGAGGTTCCGAACACTATTTAACTTGCC
TRV:NbSec5-1 ATAAGATCTGTTCAAGGAATGCTGCAGAGGTTCCGAACACTATTTAACTTGCC
TRV:NbSec5-2 ATAAGATCTGTTCAAGGAATGCTGCAGAGGTTCCGAACACTATTTAACTTGCC
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107 120 130 140 159
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160 170 180 190 200 212
TRV:Sec5 AGTACAGGAAAGCAAAGTCAATCGTTCTGCCTTCTCATGTTGGAATTTTGAAG
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TRV:NbSec5-2 AGTACAGGAAAGCAAAGTCAATCGTTCTGCCTTCTCATGTTGGAATTTTGAAG
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TRV:SlSec5-2 AGTACAGGAAAGCAAATTCAATTGTGCTGCCTTCTCATGTGGGAATTTTGAAG
213 220 230 240 250 265
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266 280 290 300 318
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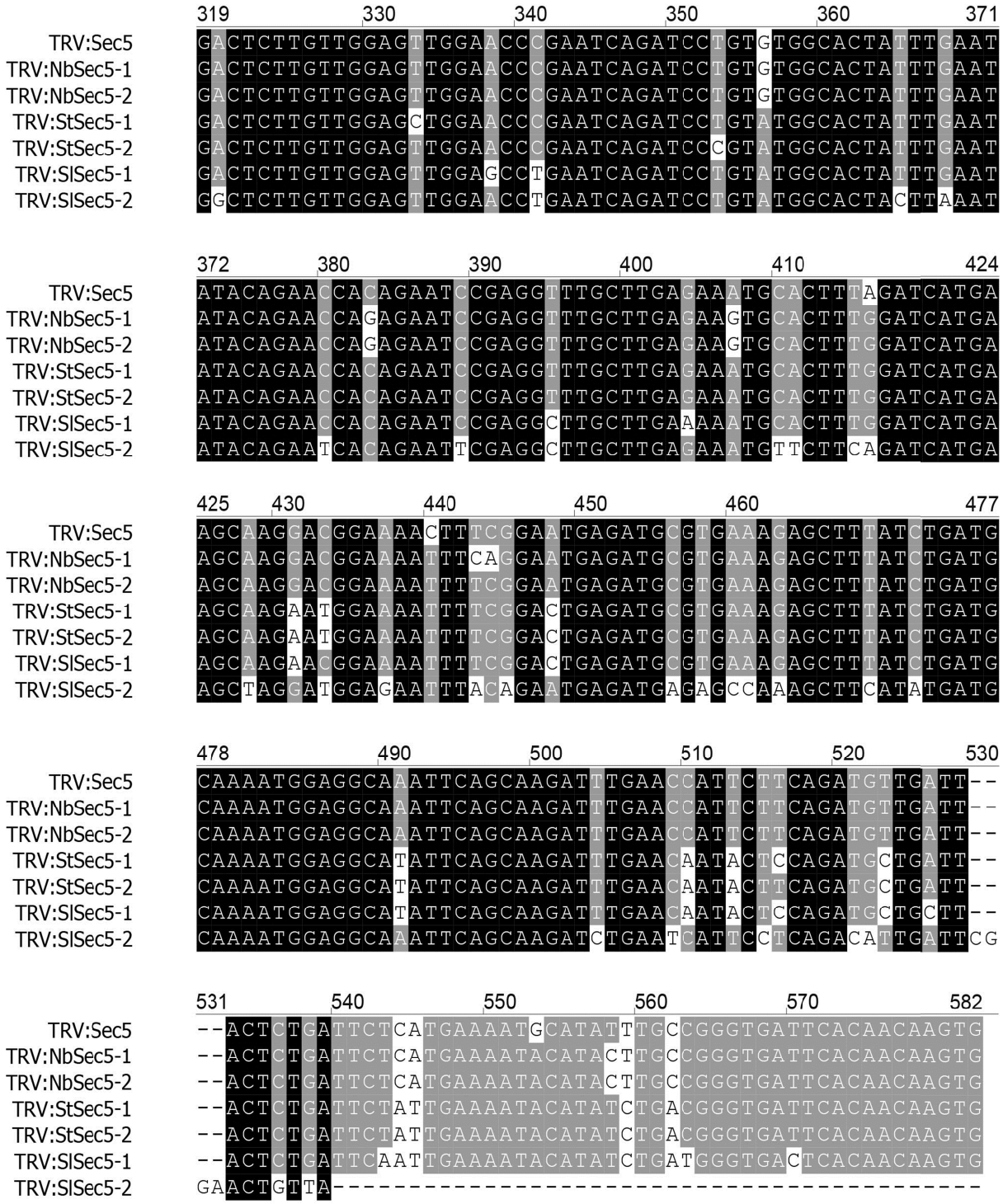

Figure S5. Alignment of the DNA fragment of 578 bp cloned in the silencing construct TRV:*Sec5* with *Sec5* cDNA sequences from potato (*StSec5-1* and *StSec5-2*), tomato (*SlSec5-1* and *SlSec5-2*), and *N. benthamiana* (*NbSec5-1* and *NbSec5-2*).

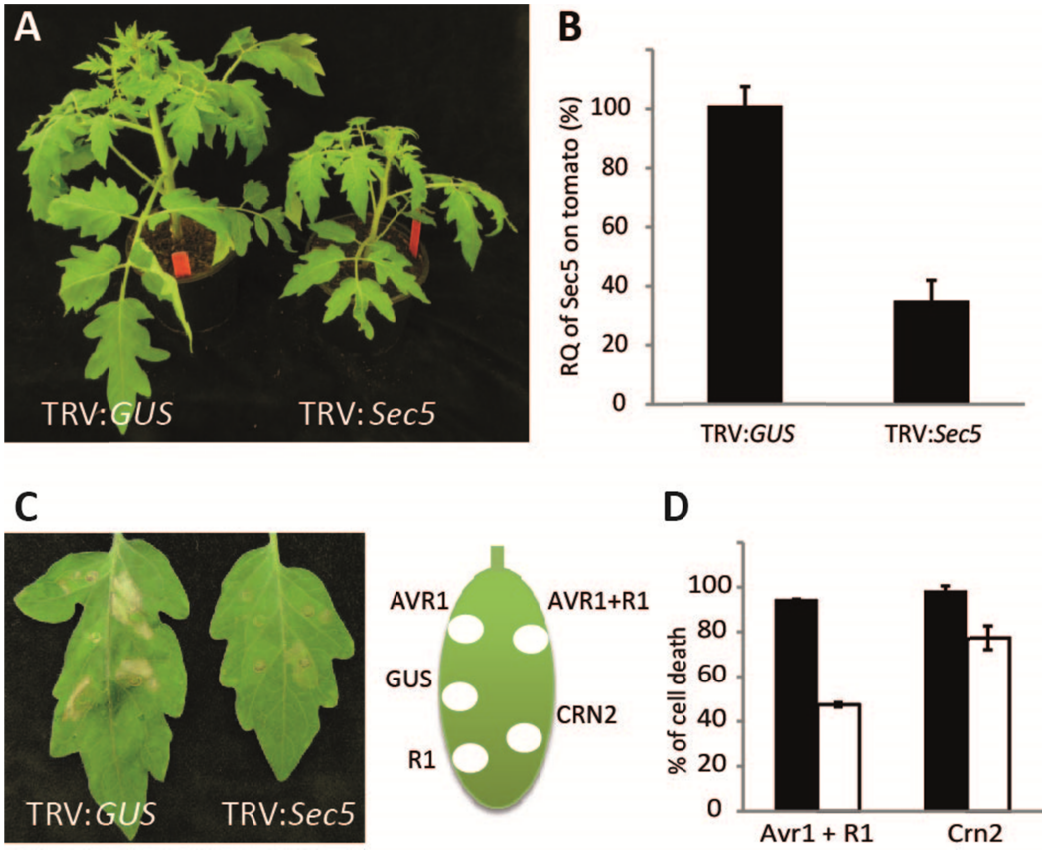

Figure S6. Cell death responses are attenuated in *Sec5*-silenced tomato plants.
(A) Phenotypes of tomato plants at 21 days after inoculation with TRV:*GUS* (control) and the silencing construct TRV:*Sec5*. (B) *Sec5* expression in tomato plants three weeks after inoculation with the TRV:*Sec5* silencing construct. Q-RT-PCR analysis was performed using *Actin* expression for normalization. On the Y-axis the Relative Quantification (RQ) of *Sec5* expression is shown with the expression in the control plants (TRV:*GUS*) set at 100%. (C) Leaves of control (TRV:*GUS*) and *Sec5*-silenced (TRV:*Sec5*) tomato plants were infiltrated with *A. tumefaciens* AGL1 harbouring *AVR1*, *R1*, *GUS*, *CRN2* and *AVR1+R1* as shown in the schematic drawing on the right. Pictures were taken at 6 dpi. (D) Graphs showing the percentage of infiltrated sites developing cell death mediated by co-agroinfiltration of *R1+AVR1* and agroinfiltration of *CRN2* at 6 dpi on control (■, TRV:*GUS*) and *Sec5*-silenced (□, TRV:*Sec5*) leaves. The graph shows the mean of three biological repeats with error bars.

Table S1. Primers used in this study.

Primer name	Sequence(5'-3')
AVR1-like-F	caccatggccggattcgaccac
AVR1-like-Rwostop	aaatggtaccacaacatgtccacc
Avr1-F	caccatgttcgaccacgacaagg
Avr1-Rwostop	aaatggtaccacaacatgtccaccaagc
R1-F	gagctcatgaatttcaacaatgaattgtctgatctg
R1-R	gcggccgcctatcttatttctgcaagaatatttttttactgc
Sec5F	caccatgtcaaccgacactgacgac
Sec5Rwistop	ttatctacgtctgggcctgg
Sec5Rwostop	tctacgtctgggcctggaaaaactcg
Sec5-vigs-F	accggatccttgctaatcgtgcttttgggccac
Sec5-vigs-R	acggagctccacttgttgtgaatcacccggcaaatatg
Sec5-RT-F	agttaagacggattgaggaagacc
Sec5-RT-R	tagtggcccaaaagcacgattagc
Nb/SlActinF	tatggaaacattgtgctcagtgg
Nb/SlActinR	ccagattcgtcatactctgcc
IPIO1-F	caccatgcgttcgctcctgttgac
IPIO1-R	gctagggccaacgttttatcgtac
IPIO4-F	caccatgcgttcgctcctgttg
IPIO4-R	gctagggccaacgtttttatcgtacc
CRN2-F	ctgagctcatggtcaagcttgtttgtgcgatc
CRN2-R	gtgcggccgctcagctgaaataactcaaagtag

Chapter 5

The C-terminus of *Phytophthora infestans* RXLR effector AVR1 determines interaction with its host target Sec5 and promotes disease susceptibility

Yu Du, Klaas Bouwmeester, Mohamed Mpina, Francine Govers

Abstract

The oomycete *Phytophthora infestans* is known as the late blight pathogen, which causes severe problems in potato production. It is able to secrete hundreds of effectors that play a role in suppressing host defense and facilitating colonization. However the mechanisms of effector-triggered host susceptibility are hardly known. AVR1 is one of the many RXLR effectors of *P. infestans*, and triggers a hypersensitive response in plants carrying the corresponding resistance gene *R1*. In a previous study *P. infestans* RXLR effector AVR1 was found to interact with the exocyst subunit Sec5 in the host cell to suppress PAMP-triggered immunity (PTI). Moreover, Sec5 was also found to be involved in HR induced by R1/AVR1 recognition. In this study, we investigated the role of Sec5 in cell death triggered by different cell death inducers. Sec5 was found to be required for INF1- and CRN2-mediated cell death, while AVR1 was determined to suppress Sec5-dependent cell death. Subsequently, using various motif deletion and chimeric constructs we investigated the conserved C-terminal motifs and regions in AVR1 that are required for Sec5 targeting and disease promotion. Our results show that AVR1 interacts with Sec5 via its C-terminal Y motif and T-region. The latter was also found to be required for the virulence functions of AVR1.

Introduction

Late blight disease caused by the oomycete plant pathogen *Phytophthora infestans* forms one of the most significant threats to potato and tomato production. *Phytophthora infestans* is able to infect its hosts by secreting a plethora of small proteins – commonly known as effectors – that modulate host defense. Well-known are the RXLR effectors, which contain a conserved N-terminal RXLR (arginine-X-leucine-arginine) motif that functions in the translocation of RXLR effectors into the host cytoplasm (Rehmany et al., 2005; Whisson et al., 2007). In contrast, the C-terminal ends of RXLR effectors, which are essential for effector functions, are very diverse (van Poppel et al., 2009; Dou et al., 2008; Bos et al., 2006). Nevertheless, bioinformatics analysis revealed three conserved motifs in C-termini of RXLR effectors (Jiang et al., 2008). These sequence motifs were named W, Y and L after their most conserved amino acid, and can occur in a repeated fashion in RXLR effectors.

Investigating the role of effectors in pathogenesis helps us to understand how pathogens colonize the host plant. However, little is known about the mechanisms that oomycete pathogens use to modulate host cell functionality, and how pathogens are recognized and activate plant immunity. Thus far, several RXLR effectors have been assigned to suppress host defense responses at different levels to enable parasitic infection (Bos et al., 2006; Oh et al., 2009; Halterman et al., 2010, Anderson et al., 2012). For instance, several RXLR effectors are able to suppress host basal defense by disrupting downstream MAPK signalling pathways of pathogen-associated molecular pattern (PAMP) triggered immunity (PTI) (Cabral et al., 2011, Anderson et al., 2012; King et al., 2014; Zheng et al., 2014; Fabro et al., 2011). In addition, RXLR effectors can evade effector-triggered immunity (ETI), as reported for *P. infestans* effector IPI-O4 that suppresses Rpi-blb1-mediated resistance (Halterman et al., 2010) and for PsAvh163 from *Phytophthora sojae* that subverts ETI mediated by the soybean NLR RPS4 (Anderson et al., 2012). RXLR effectors are also shown to promote *Phytophthora* proliferation by multiple other strategies. For example, the *P. sojae* RXLR effector AVR3b acts as an NADH and ADP-ribose pyrophosphorylase (Dong et al., 2011), and *P. infestans* effector Pi03192 targets host transcription factors (McLellan et al., 2013) to modulate plant immunity. In addition, *P. infestans* RXLR effector IPI-O was found to disturb host cell wall integrity to facilitate pathogen proliferation (Bouwmeester et al., 2011).

Secretory pathways play vital roles in host immunity by secreting antimicrobial compounds to the infection site, either to kill the pathogen directly, or to reinforce physical barriers that halt pathogen invasion (Frey and Robatzek, 2009; Robatzek, 2007). Plasma membrane syntaxins, which function at the last step in vesicle secretion, were found to play a role in plant defense against pathogen invasion, e.g. NbSYP132 was shown to be required for secretion of the host defense related protein PR1, whereas another SNARE protein PEN1 is needed for papillae formation and focal secretion (Kalde et al., 2007; Collins et al., 2003). Also the exocyst complex, which works one step upstream of SNAREs in the plant secretory system, appears to be involved in plant defense, as one of its subunits, the exocyst component Exo70B2, was shown to be important for localized cell wall apposition and PTI (Pecenkova et al., 2011; Stegmann et al., 2012). Hence, it can be hypothesized that plant pathogen effectors potentially target vesicle trafficking-related proteins to manipulate host focal secretion in order to pave the way for colonization (Nomura et al., 2011; Nomura et al., 2006; Ham et al., 2006; Driouich et al., 1997; Nielsen et al., 2012). One example is the bacterium type III effector HopM1 that degrades a key factor in vesicle formation to halt host focal secretion to support infection (Nomura et al., 2011; Nomura et al., 2006). Similarly, *Phytophthora* RXLR effectors are found to suppress host focal secretion, e.g. by preventing secretion of the plant immune protease C14 (Bozkurt et al., 2011) or by targeting vesicle-related proteins, as shown for AVR1 that interacts with the host exocyst component Sec5 (**Chapter 4**).

The *P. infestans* RXLR effector AVR1 is highly homologous in protein sequence with AVR1-like (A-L), and both effectors contain two W motifs and one Y motif in their C-termini. In contrast to A-L, AVR1 has a 38 amino acids extension at its C-terminus, which was baptized as the T-region (**Chapter 2**). As shown previously, AVR1 interacts with the host exocyst component Sec5 to interfere with host focal secretion and to facilitate *P. infestans* colonization, whereas A-L is unable to bind with Sec5. R1/AVR1-triggered HR was found to be dependent on Sec5, while HR triggered by the R/AVR pairs Rx/CP and R3a/AVR3a appeared to be independent of Sec5 (**Chapter 4**). The aim of this study was to investigate whether Sec5 is required for cell death induced by the *P. infestans* elicitors INF1 and CRN2, and whether AVR1 is able to suppress Sec5-dependent cell death. In addition, we questioned whether AVR1 and A-L act as virulence determinants of *P. infestans* during pathogenesis on

N. benthamiana and which conserved motifs or regions of AVR1 are required for interaction with the host target Sec5 for promoting disease susceptibility.

Results

P. infestans AVR1 and A-L act as virulence factors

To test whether *AVR1* and *AVR1-like* (*A-L*) contribute to *P. infestans* colonization, we transiently expressed the effector genes in *N. benthamiana* leaves via agroinfiltration, and subsequently performed infection assays. Detached leaves of *N. benthamiana* plants transiently expressing AVR1-myc, A-L-myc or GUS were inoculated with zoospores from *P. infestans* isolate 88069. Lesion diameters were measured at 6 days after inoculation (dai) and lesion areas were calculated. As shown in Figure 1, the presence of AVR1 or A-L resulted in larger lesions, which suggests that both RXLR effectors induce *Phytophthora* proliferation *in planta*, and as such serve as genuine virulence factors.

To investigate which motif of AVR1 is required to promote *P. infestans* colonization, infection assays with *P. infestans* were performed on *N. benthamiana* leaves that transiently express AVR1 deletion and chimeric constructs, which were previously described in **Chapter 2**. Obtained results (Figure 1) indicate that the T-region in the C-terminus of AVR1 is needed to promote *P. infestans* colonization. This is shown by the fact that AVR1-ΔT (i.e. AVR1 without the T-region) loses its ability to promote *Phytophthora* growth, while the T-region alone is sufficient to promote *P. infestans* colonization. In addition, a chimeric version consisting of A-L fused with the T-region of AVR1 was as well found to promote *Phytophthora* growth.

The T-region of AVR1 contains no known domain or motif and shows no similarity with any other known proteins. Since AVR1 was shown to interact with the host exocyst subunit protein Sec5 to suppress plant basal defense (**Chapter 4**), we hypothesised that the T-region may be required for Sec5 interaction. A-L which lacks the T-region is also able to promote *P. infestans* colonization but does not interact with Sec5 (**Chapter 4**). Hence, it is possible that A-L promotes host colonization through a different mechanism.

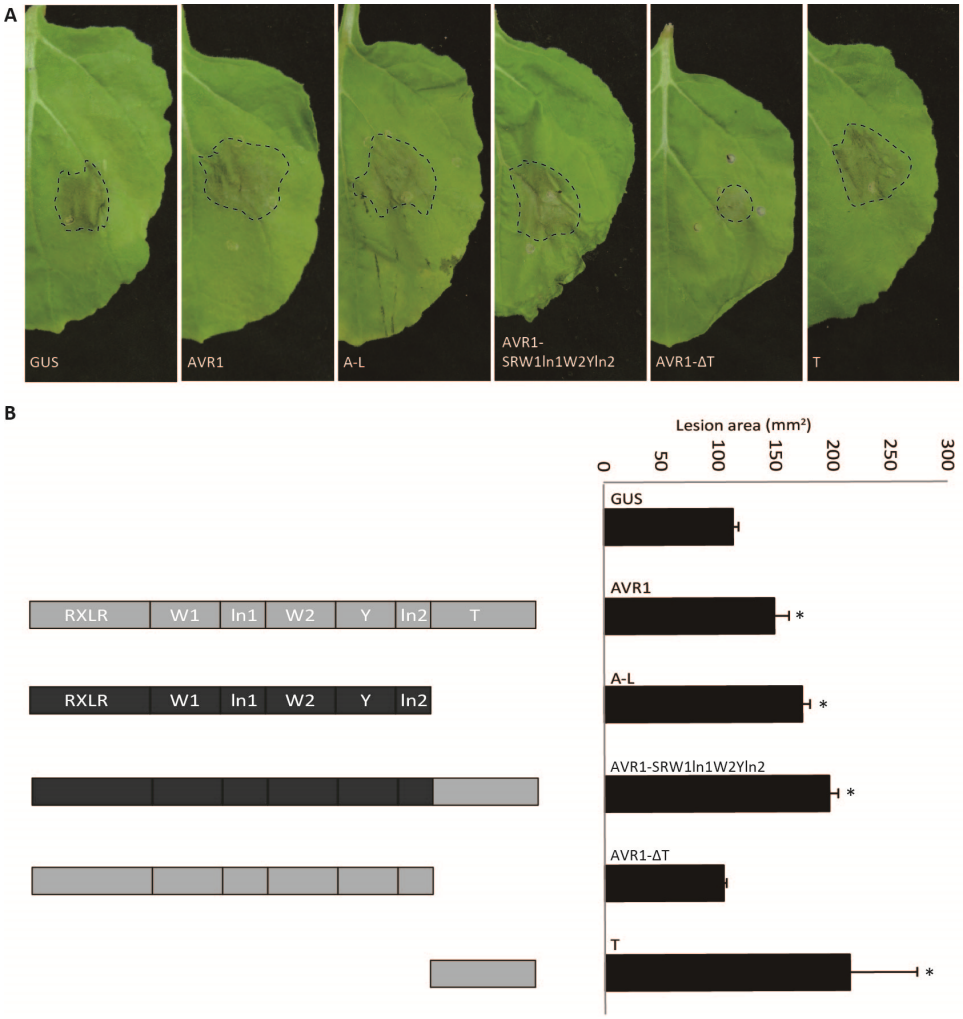

Figure 1. The T-region of AVR1 is required to promote colonization of *P. infestans*. (A) Lesions on *N. benthamiana* leaves transiently expressing GFP, AVR1, A-L or chimeric and truncated AVR1 versions. Dotted lines indicate lesion areas at 6 days after inoculation (dai). (B) Mean lesion areas at 6 dai with error bars representing standard deviations. Asterisks indicate significant differences, (N⩾8, one-sided Student's t-test, P<0.05). Experiments were repeated three times with similar results.

AVR1, but not A-L, suppresses Sec5-dependent cell death

To investigate the role of Sec5 in elicitor-triggered cell death, we transiently expressed INF1, CRN2 and BAX into *Sec5*-silenced *N. benthamiana* leaves via agroinfiltration. At 5 days post infiltration (dpi), both INF1- and CRN2-triggered cell death was found to be reduced in *Sec5*-silenced plants compared to control plants (TRV:GUS-treated). Less than 60 percent of the infiltrated sites developed cell death indicating that cell death triggered by INF1 and CRN2 is Sec5-dependent (Figure 2A).

In contrast, BAX-triggered cell death is not reduced in *Sec5*-silenced plants, and thus independent of Sec5.

To determine to what extent AVR1 is modulating Sec5-dependent elicitor-triggered cell death responses, AVR1 was co-agroinfiltrated with the cell death elicitors INF1, CRN2 and BAX. Cell death symptoms were scored at 4 dpi. Leaf areas that co-express INF1 or CRN2 in combination with either GUS or A-L developed cell death responses in almost all infiltrated sites (>80%), but when co-expressed with AVR1, the percentage of infiltrated sites that developed elicitor-triggered cell death was less than 60%. BAX-induced cell death was neither affected by AVR1 nor A-L (Figure 2B). This indicates that suppression of cell death by AVR1 is mediated by its interaction with Sec5. This was further supported by the finding that AVR1 was not able to modulate other Sec5-independent processes (Figure 2C). As previously shown (**Chapter 4**), HR elicited by co-expressing the R/AVR pairs R3a/AVR3a and Rx/CP is not reduced in *Sec5*-silenced plants. Here we show that also co-expression of these R/AVR pairs with AVR1 does not change the HR response when compared to the control (Figure 2C). Similarly, the AVR1 homologue A-L did not show cell death suppression activity in any of the tested cases. Taken together these results show that AVR1 is able to suppress Sec5-dependent INF1- and CRN2-induced cell death but not any of the tested Sec5-independent cell death processes, and support the hypothesis that AVR1 manipulates Sec5 function to promote *P. infestans* colonization.

AVR1 suppresses CRN2-induced cell death via its T-region

In order to determine which motif or region in AVR1 is responsible for cell death suppression, AVR1, A-L and various chimeric and deletion constructs were co-expressed with CRN2 in *N. benthamiana* leaves. At 4 dpi, more than 80% of the GUS/CRN2 co-agroinfiltrated sites developed cell death. Similarly, co-agroinfiltration of CRN2 with either A-L or AVR1-ΔT sites developed comparable levels of cell death. In contrast, less than 50% of sites that co-express CRN2 with AVR1, AVR1-SRW1ln1W2Yln2 or T developed cell death (Figure 3). These results confirm that CRN2-induced cell death is not suppressed by A-L, and point to the importance of the T-region of AVR in suppressing cell death. Results obtained from assays in which we co-expressed CRN2 with various AVR1 or A-L deletion and chimeric constructs revealed that neither the W1, W2 and Y motifs nor the linker

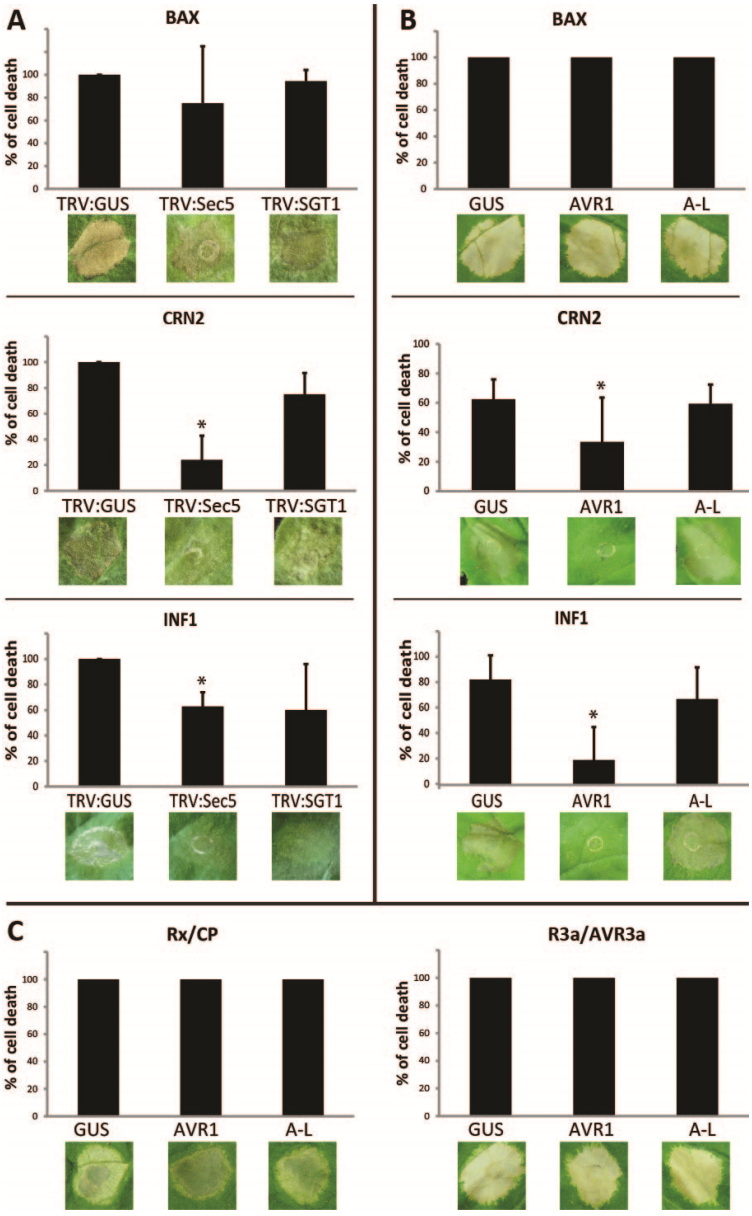

Figure 2. AVR1 suppresses Sec5-dependent elicitor-triggered cell death.
(A) *GUS*-, *Sec5*- and *SGT1*-silenced *N. benthamiana* leaves agroinfiltrated with BAX, CRN2 or INF1. Cell death was scored and pictures were taken at 5 days post infiltration (dpi). Graphs show the average percentage of infiltrated sites developing cell death from at least two biological replicates. (B) AVR1, GUS or A-L co-agroinfiltrated with BAX, CRN2 or INF1 in *N. benthamiana* leaves. Cell death development was observed and pictures were taken at 4 dpi. Graphs show average percentages of infiltrated sites that developed cell death obtained from two biological replicates. (C) *N. benthamiana* leaves co-agroinfiltrated with AVR1 and Rx/CP or R3a/AVR3a. Graphs show average percentages of infiltrated sites that developed cell death obtained from two biological replicates. Significant differences are indicated by asterisks ($N \geqslant 9$, one-sided Student's t-test, $P<0.05$).

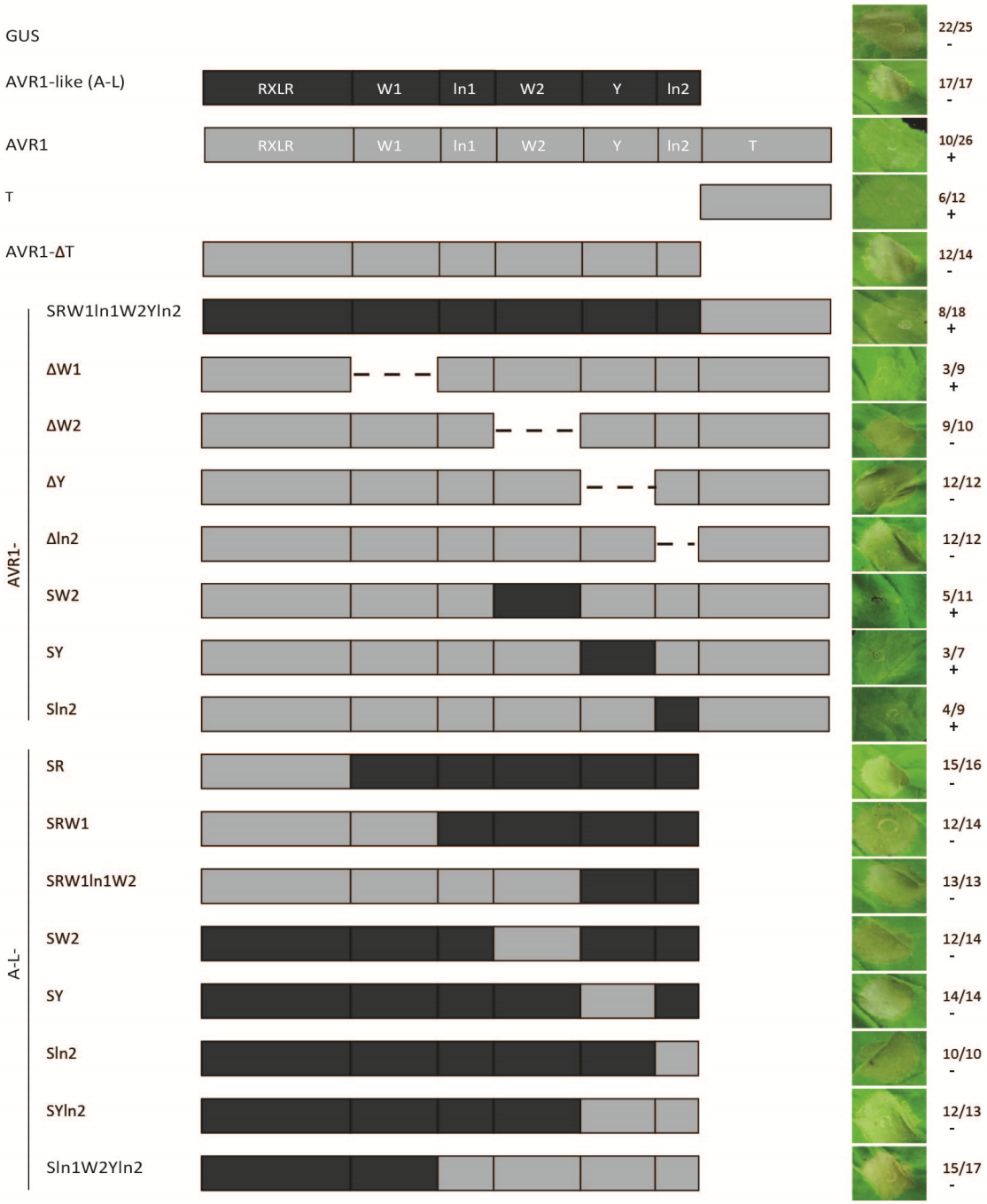

Figure 3. The T-region of AVR1 is sufficient to suppress CRN2-induced cell death. *Agrobacterium* stains harboring AVR1 deletion and chimeric constructs were co-agroinfiltrated with CRN2 into *N. benthamiana* leaves and cell death was monitored at 4 dpi. On the left, a schematic representation of AVR1, A-L and the used motif deletion and chimeric constructs is shown. On the right, the observed responses and numbers of infiltrated sites that developed cell death versus the total number of infiltrated sites are shown (+: suppression; -: no suppression).

regions are required to suppress CNR2-induced cell death, and that only constructs which contain the T-region are able to suppress cell death. Deletion of W2, Y or ln2 may lead to protein misfolding (**Chapter 2**), and this may explain why despite the fact that these constructs contain the T-region, there is no cell death suppression observed. It can be concluded that (i) the W and Y motifs of AVR1 are not required to suppress CRN2-induced cell death, and that (ii) the T-region of AVR1 is the pivotal determinant for suppression of CRN2-induced cell death.

The T-region and Y motif of Avr1 are required for Sec5 interaction

As described in **Chapter 4**, AVR1 is able to bind with the host protein Sec5 in a yeast two-hybrid (Y2H) interaction assay, whereas A-L is not. To further verify which part of AVR1 is important for the interaction with Sec5, additional Y2H assays were performed. Various chimeric and deletion constructs were co-transformed with Sec5 in yeast, and analysed for growth on selection medium. As shown in Figure 4, a yeast transformant containing a version of AVR1 that lacks the 38 amino acid long T-region at its C-terminus (AVR1-ΔT) is unable to grow on selection medium, indicating that it lost its ability to bind Sec5. However, the T-region by itself also failed to interact with Sec5 (Figure 4). Also the chimeric protein A-L-ST, in which the T-region of AVR1 is fused to A-L, is not able to bind Sec5 (Figure 4), suggesting that other motifs of AVR1 are required for the physical interaction. To investigate this, we analysed chimeric proteins in which motifs were exchanged from AVR1 to A-L and *vice versa* (Figure 4). The results show that the N-terminal RXLR region, and both W motifs of AVR1 are not required for Sec5 interaction. However, when the Y motif of AVR1 is replaced by the Y motif of A-L the interaction is lost, suggesting that – in addition to the T-region – the Y motif of AVR1 is also required for binding Sec5 (Figure 4).

Discussion

Increasing evidence shows that suppression of innate immunity and cell death responses are important activities of effectors secreted by oomycete and fungal plant pathogens to promote host colonization (Bouwmeester et al., 2011; Dou et al., 2008; Bos et al., 2006; van der Does et al., 2008). Previously, we have reported that the *P. infestans* RXLR effector AVR1 targets the exocyst component Sec5 to

suppress plant immunity, and that Sec5 plays a role in plant defense against *P. infestans* and in downstream signalling of R1-mediated immunity (**Chapter 4**).

In this study, we firstly showed that AVR1 and its close homolog A-L are virulence factors as both of them can promote *P. infestans* colonization when transiently expressed in *N. benthamiana* leaves. We extended our study of the role of Sec5 in cell death responses triggered by different cell death elicitors, i.e. the *P. infestans* PAMP INF1 and effector CRN2, as well as the mouse programmed cell death protein BAX. Results show that Sec5 is required for cell death mediated by INF1 and CRN2, but not by BAX. As shown in **Chapter 4**, R1/AVR1-mediated HR was dependent on Sec5, while R3a/AVR3a- and Rx/CP-mediated HR were not. We thus investigated whether AVR1 is able to suppress Sec5-independent HR. HR triggered by recognition of effectors by NLR proteins was neither affected by *Sec5*-silencing, nor by co-expression of AVR1. Taken together, these data suggest that AVR1 only suppresses Sec5-dependent cell death. In contrast, the AVR1 homolog A-L that does not target Sec5 was found unable to suppress both Sec5-dependent and independent cell death.

The plant exocyst is known to participate in the recycling of plasma membrane receptors, such as the auxin efflux carriers PIN1 and PIN2, and the brassinosteroid receptor BRI1 (Drdova et al., 2013). Hence, it can be hypothesized that pathogen-recognition receptors (PRRs) that function in PTI are also dependent on the exocyst for localizing on the host plasma membrane. The *P. infestans* PAMP INF1 was recently shown to be recognized by the potato plasma membrane receptor ELR (Du, 2014). Hence, we assume that reduction of INF1-induced cell death upon expression of AVR1 and *Sec5*-silencing in *N. benthamiana* could be due to the fact that ELR-like receptors are hampered in their localization towards the plasma membrane. Although CRN2 is known to localize inside the host nucleus (Schornack et al., 2010), the mechanisms on how CRN2 is able to trigger necrosis are unknown. It could be well possibly that Sec5 functions in downstream signalling of CRN2-induced necrosis.

Finally, we investigated the conserved C-terminal motifs or regions of the RXLR effector AVR1 for their role in suppression of CRN2-induced cell death and Sec5 binding. This revealed that (i) the T-region of AVR1 is essential for maintaining its virulence activities, and (ii) both the Y motif and T-region determine the direct physical interaction of AVR1 with Sec5. RXLR effectors share a conserved N-terminal

RXLR motif that is required for effector host translocation and that is followed by a diverse C-terminal effector domain determining effector functionality. As reported by Jiang et al (2008), C-termini of more than half of the analysed RXLR effectors contain one or more of these conserved motifs, which are known as the W, Y and L motifs. Thus far a few similar studies have been performed, and all showed that the virulence and avirulence activities of RXLR effectors are determined by their C-terminal domains. For instance, the C-terminal half of *P. infestans* RXLR effector AVR3a is responsible for triggering R3a-mediated HR and suppressing INF1-mediated cell death (Bos et al., 2006). The W and Y motifs of *P. sojae* AVR1b were identified to be critical for suppression of BAX-induced cell death and activation of the soybean resistance protein Rps1b (Dou et al., 2008). In addition, Dou et al. (2008) showed that also other RXLR effectors containing multiple W, Y or L motifs in their C-termini, including *P. sojae* Avh331 and Avh163 and the *Hyaloperonospora arabidopsidis* effector RXL96, are able to suppress BAX-triggered cell death. However, *P. sojae* effector AVR4/6, which lacks any C-terminal conserved motif, was not able to suppress BAX-induced cell death, which indicates that these conserved motifs play a role in suppression of cell death (Dou et al., 2008). In **Chapter 2** we have identified that there are two epitopes in AVR1 that trigger R1-mediated HR, one being the C-terminal region that contains the conserved W1, W2 and Y motifs, while the other is the T-region. In this study by using motif deletion and chimeric constructs we observed that the T-region of AVR1 is essential and sufficient for promoting *P. infestans* colonization and suppression of CRN2-induced cell death. However, the conserved motifs in AVR1 are essential for proper folding of the protein (or its T-region), as deletion of W2, Y or ln2 abolished the suppression of cell death. This is confirmed by the fact that A-L contains the same motifs as AVR1 but is not able to suppress CRN2-induced cell death, while fusion with the T-region enabled A-L to suppress cell death.

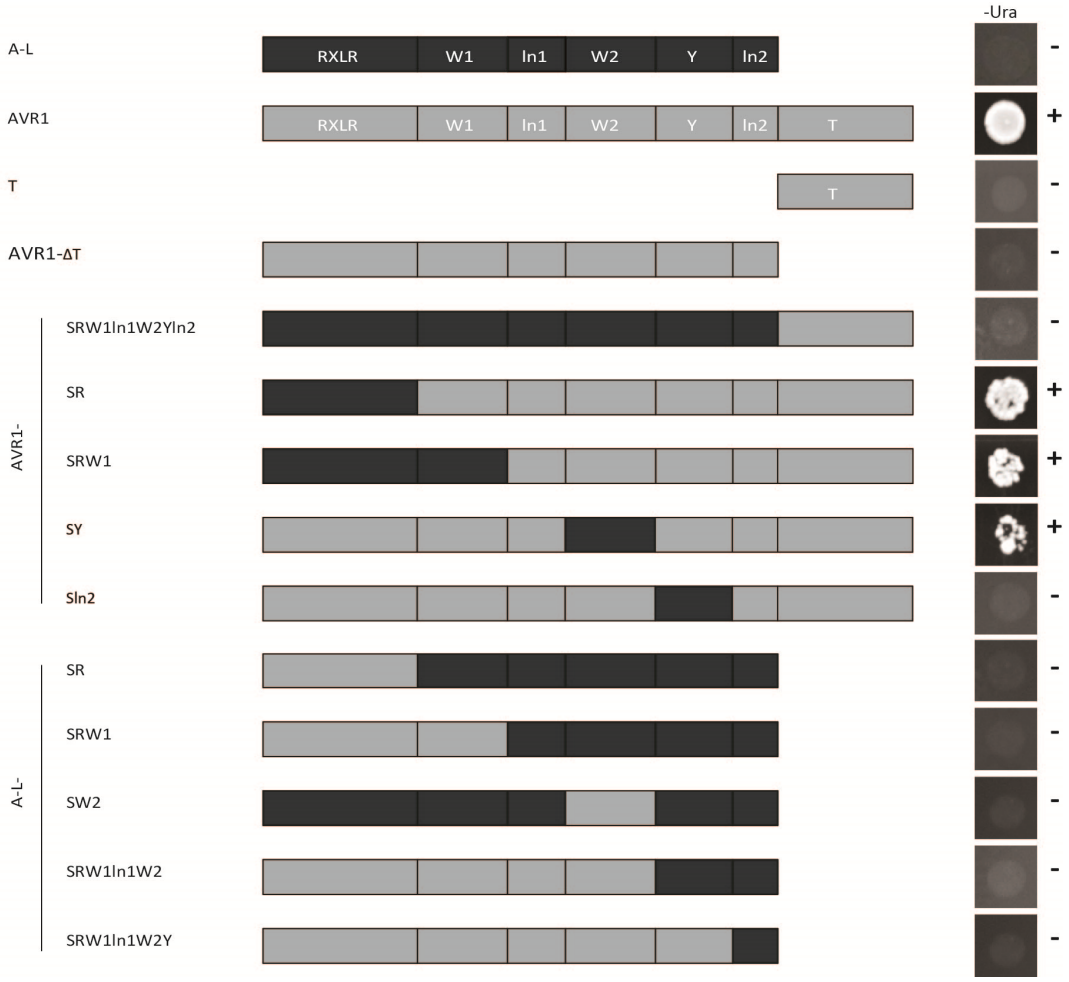

Figure 4. The T-region of AVR1 is required, but not sufficient for interaction with Sec5 in yeast.
The left panel shows the schematic representation of AVR1, A-L and various motif deletion and chimeric derivatives. The right panel shows pictures of yeast colonies on URA-deficient SD medium after 3 days of incubation (+; colony growth, -; no growth).

Since AVR1 only suppresses Sec5-dependent cell death, we investigated whether the T-region is the determinant for Sec5 interaction by Y2H assays. As shown in Figure 4, the T-region alone is not sufficient for interaction with Sec5, nor is AVR1-ΔT. The chimeric constructs show that the RXLR, W1, W2 motifs of AVR1 are all not essential for Sec5 interaction as replacing them by the corresponding motifs from A-L does not inhibit the interaction of the chimeric proteins with Sec5. However, when the AVR1 Y motif is replaced with the Y motif from A-L the interaction is abolished, which indicates that the Y motif of AVR1 is required for Sec5 interaction.

Taken together, both the Y motif and the T-region of AVR1 are needed for Sec5 interaction.

The inability of A-L to interact with Sec5 does not hamper the virulence activity of A-L; this is likely due to the fact that effector proteins could have multiple target proteins for modulating host immunity. For instance, the bacterial effector HopM1 of *Pseudomonas syringae* not only to binds with the ARF-GEF protein AtMIN7 to inhibit plant secretion of antimicrobial compounds, but also interacts with a host 14-3-3 protein to manipulate PAMP-triggered oxidative burst and stomatal immunity (Nomura et al., 2011; Lozano-Durán et al., 2013). In addition the *P. infestans* RXLR effector AVR3a, which stabilizes the host U-box E3 ligase CMPG1 to promote pathogen colonization, is also able to bind the exocyst component Sec3 in yeast (Bos et al., 2010). Hence, it can be postulated that AVR1 and A-L are able to target other host proteins in order to facilitate *P. infestans* colonization.

Material and methods

Plasmid construction

Plasmids used for *in planta* assays were constructed as described in **Chapter 2** and **Chapter 4** of this thesis. These same plasmids were used as templates for PCR amplification with the primer pairs listed in Supplemental Table 1. The obtained fragments were cloned into the vector pENTR/D-TOPO and subsequently the entry vectors containing the *AVR1* and *A-L* chimeric genes were recombined into the yeast two-hybrid prey vector pDEST32. The construction of the bait vector pDEST22:Sec5 is described in **Chapter 4**.

Agroinfiltration assays

Recombinant *Agrobacterium tumefaciens* strains were grown in YEB with appropriate antibiotics for about 24 hours at 28 °C. *Agrobacterium* cultures were centrifuged for 10 min, and the pellets were resuspended in infiltration buffer (10 mM MES pH 5.6, 5 g/l MS salts (w/o vitamins), 20 g/l sucrose, and 150 µM acetosyringone) to an optical density (OD_{600}) of 0.6 prior to agroinfiltration into 4-week-old leaves of *Nicotiana benthamiana*. Co-agroinfiltration was performed with mixtures of *Agrobacterium* suspensions in 1:1 (for infection assays) or 2:1 (2 suppressor:1 cell death inducer for suppression assays) volume ratios.

Pathogen inoculation

P. infestans isolate 88069 was grown on rye sucrose agar medium at 18°C for 10 days. Zoospores were isolated according to Champouret et al. (2009). *N. benthamiana* leaves harvested 1 day after agroinfiltration were inoculated at the abaxial sides with 10 µl droplets of a *P. infestans* zoospore suspension ($1*10^5$ zoospores/mL). Inoculated leaves were incubated at high humidity at 18 °C, with the first 24 hours in the dark. Lesion diameters were measured 6 days after inoculation (dai), and lesion areas were determined as described by Vleeshouwers et al, (1999).

Yeast-two hybrid assays

Plasmid pDEST22:StSec5 (**Chapter 4**) was co-transformed with pDEST32 prey plasmids containing *AVR1*, *A-L* or the various derivatives in yeast strain MaV203 using the ProQuest Two-Hybrid System (Invitrogen). Yeast co-transformants were selected on SD medium lacking the amino acids Trp and Leu (SD-WL), and subsequently the interaction was monitored via selection on Ura-deficient SD medium (SD-WLU). Yeast controls to determine interaction were provided by the ProQuest Two-Hybrid System (Invitrogen).

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Supplementary information

Table S1. Primers used in this study.

Primer name	Forward primer sequence	Reverse primer sequence
AVR1	caccatgttcgaccacgacaagg	ttaaaatggtaccacaacatgtcc
A-L	caccatggccggattcgaccac	tcacagcccctccaacttgtc
Avr1-ΔW1	caccatgttcgaccacgacaagg	ttaaaatggtaccacaacatgtcc
Avr1-ΔW2	caccatgttcgaccacgacaagg	ttaaaatggtaccacaacatgtcc
Avr1-ΔY1	caccatgttcgaccacgacaagg	ttaaaatggtaccacaacatgtcc
A-L-SR	caccatgttcgaccacgacaagg	tcacagcccctccaacttgtc
T	caccatgttaagtggaggatcacctgag	ttaaaatggtaccacaacatgtcc
A-L-SRW1	caccatgttcgaccacgacaagg	tcacagcccctccaacttgtc
AVR1-SYln2ΔT	caccatgttcgaccacgacaagg	tcacagcccctccaacttgtc
AVR1-ΔT	caccatgttcgaccacgacaagg	tcacagcccgtgccactgatc
AVR1-SR	caccatggccggattcgaccac	ttaaaatggtaccacaacatgtcc
AVR1-SRW1	caccatggccggattcgaccac	ttaaaatggtaccacaacatgtcc
A-L-ST	caccatggccggattcgaccac	ttaaaatggtaccacaacatgtcc
AVR1-SW2	caccatgttcgaccacgacaagg	ttaaaatggtaccacaacatgtcc
AVR1-SY	caccatgttcgaccacgacaagg	ttaaaatggtaccacaacatgtcc
A-L-Sln1W2Yln2	caccatggccggattcgaccac	ttaaaatggtaccacaacatgtcc
A-L-SW2	caccatggccggattcgaccac	tcacagcccctccaacttgtc
A-L-SYln2	caccatggccggattcgaccac	tcacagcccgtgccactgatc
A-L-SY	caccatggccggattcgaccac	tcacagcccctcccactgatc
AVR1-Sln2	caccatgttcgaccacgacaagg	ttaaaatggtaccacaacatgtcc
A-L-Sln2	caccatggccggattcgaccac	taacagcccgtgcaacttgtc
AVR1-Δln2	caccatgttcgaccacgacaagg	ttaaaatggtaccacaacatgtcc
AVR1-Sln2ΔT	caccatgttcgaccacgacaagg	tcacagcccctcccactgatc
pDEST22/32	tataacgcgtttggaatcactacagg	gacaaccttgattggagacttgacc
M13	cgttgtaaaacgacggccag	tgccaggaaacagctatgacc

Chapter 6

Identification of Solanaceous exocyst subunits and their role in defense against *Phytophthora* pathogens

Yu Du, Klaas Bouwmeester, Jeroen Berg, Francine Govers

Abstract

The exocyst, a multi-protein complex consisting of eight subunits, plays an essential role in many biological processes by mediating secretion of post-Golgi derived vesicles towards the plasma membrane. In contrast to yeast and animals, plants have two or more copies of most exocyst subunit genes and a largely expanded gene family encoding exocyst subunit Exo70. In recent years roles for plant exocyst subunits in pathogen defense have been uncovered, largely based on studies in the model plant Arabidopsis. Fairly few studies have been undertaken to assign the role of exocyst subunits in plant defense in other plants species, including crops. In this study, predicted protein sequences from exocyst subunits were retrieved by mining databases from the Solanaceous plants *Nicotiana benthamiana*, tomato and potato. Subsequently, their evolutionary relationship with Arabidopsis exocyst subunits was analysed and their role in plant defense against *Phytophthora* pathogens was investigated. Virus-induced gene silencing in *N. benthamiana* and tomato showed that all exocyst subunits are required for proper plant defense against *Phytophthora infestans* and *Phytophthora capsici*, with the exception of a few Exo70 paralogs. Most of the exocyst subunits are also involved in plant callose deposition. This study provides a first indication that multiple exocyst components of Solanaceous plants are functioning in immunity against *Phytophthora* pathogens.

Introduction

The exocyst, an evolutionary conserved multi-protein that consists of eight subunits, is largely involved in directing post-Golgi-derived vesicles towards the plasma membrane (Munson and Novick, 2006). This process works one step before docking and fusion of vesicles at the membrane mediated by the soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment receptor complex, known as the SNARE-complex (Duman and Forte, 2003). The exocyst was first discovered in yeast and shown to be required for secretion and polarized growth during budding (Terbush and Novick, 1995). Later on homologs of the eight exocyst subunits, i.e. Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84, were identified in higher organisms (TerBush et al., 1996; Hsu et al., 1996). In yeast and mammals interaction between the individual exocyst subunits has been studied by different methods, and this revealed that each exocyst subunit can associate with multiple other subunits in the complex (Munson and Novick, 2006). Studies using chromatographic fractionation and yeast two-hybrid showed that also in plants the exocyst is a true protein complex, and that the pairwise interactions between the exocyst subunits are conserved resembling those in yeast and animals (Hala et al., 2008; Zhang et al., 2013). Unlike yeast and animals, which have only one gene for each exocyst subunit, some of the exocyst subunits in plants are encoded by two genes or even more i.e. Arabidopsis has three Exo84 and 23 Exo70 paralogs (Cvrckova et al., 2001; Chong et al., 2010). So far, our insight into the role of the exocyst in plants is rather limited but it was suggested to play a role in growth and development, such as cytokinesis, cell plate maturation and morphogenesis (Fendrych et al., 2010; Hala et al., 2008). In Arabidopsis AtExo70A1 was shown to be required for root hair tip growth (Synek et al., 2006) and pollen tube penetration through the stigmatic surface (Samuel et al., 2009), while AtSec5, AtSec6, AtSec8 and AtSec15 appear to be required for pollen germination (Cole et al., 2005; Hala et al., 2008), and AtSec3 for embryo development (Zhang et al., 2013).

Several studies show that in plants vesicle-trafficking plays an important role in defense against pathogens by accommodating secretion of antimicrobial compounds to infection sites to hamper pathogen colonization. For example, infection of barley with the powdery mildew fungus *Blumeria graminis* leads to cell wall-associated defense accompanied with proliferation of multi-vesicular bodies (MVBs) that were shown to participate in deposition of cell wall appositions and

accumulation of H_2O_2 at the cell periphery to block fungal penetration (An et al., 2006). In addition, penetration resistance of Arabidopsis to *Blumeria graminis* was shown to be mediated by the syntaxin SYP121, a component of the SNARE-complex (Collins et al., 2003). Moreover, induction of systemic acquired resistance was reported to be dependent on NPR1-regulated expression of several secretion-related genes (Wang et al., 2005). Also exocyst complex subunits, which work one step upstream of the SNAREs in exocytosis, appear to be required for proper plant defense. The barley Exo70F-like, and Arabidopsis Exo70B2 and Exo70H are required for resistance against *B. graminis* (Ostertag et al., 2013; Pecenkova et al., 2011) and the Arabidopsis Exo70B1 is involved immunity against different pathogens (Stegmann et al., 2013). Exo70B2 – the target of the ubiquitin ligase PUB22 – was found to act as a negative regulator of plant defense elicited by pathogen-associated molecular patterns (PAMPs) (Stegmann et al., 2012) and to be required for proper papillae formation. Pecenkova et al. (2011) found that the Arabidopsis Exo70B2 mutants show aberrant papillae with halos and enhanced penetration upon *B. graminis* inoculation. Next to Exo70B2, also the SNARE syntaxin PENETRATION1 (PEN1) is required for timely assembly of papillae and focal secretion, and thus for blocking penetration of the powdery mildew fungus (Collins et al., 2003). Penetration resistance is often conferred by cell wall thickening, of which callose is a major component. Taken together these findings suggests that polarized secretion, which is mediated by the exocyst complex and regulated by small GTPases, plays an important role in callose deposition and papillae formation at the infection sites (Hsu et al., 2004; Ellinger et al., 2014).

The importance of vesicle trafficking in resistance is also shown by the fact that plant pathogens secrete effectors that target vesicle-trafficking related proteins. For example, the bacterial pathogen *Pseudomonas syringae* secretes HopM1, an effector that destabilizes AtMIN7, a key regulator of vesicle formation thereby facilitating infection of Arabidopsis (Nomura et al., 2011; Nomura et al., 2006), the fungal plant pathogen *Alternaria carthami* secretes the phytotoxin Brefeldin A (BFA) that inhibits Golgi-derived vesicle formation (Driouich et al., 1997), and the oomycete *Phytophthora infestans* uses the RXLR effector AVR1 to target the exocyst component Sec5 resulting in suppression of host defense (**Chapter 4**).

In general the roles of the exocyst subunits in plant defense are understudied. The knowledge obtained so far is rather fragmented and only covers three out of 23 Exo70 paralogs and none of the other exocyst components. The purpose of the current study was to investigate the role of the individual exocyst subunits of *Nicotiana benthamiana* and tomato in defense against *Phytophthora* pathogens. Genes encoding the exocyst subunits were identified and phylogenetic analyses were performed to study the relationship with exocyst subunits in Arabidopsis. The roles of various exocyst subunits in plant defense were studied by knocking down expression of the genes in *N. benthamiana* and tomato using virus-induced gene silencing (VIGS). Here we provide a first indication that multiple exocyst subunits are functional in resistance against *Phytophthora* pathogens and play a role in callose deposition.

Results and discussion

Identification and phylogenetic analyses of exocyst subunits from Solanaceous plants.

To identify the exocyst subunit genes of the Solanaceous plants *Nicotiana benthamiana,* potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) BLAST analyses were performed using protein sequences of Arabidopsis exocyst subunits against the predicted proteomes of *Nicotiana benthamiana*, potato and tomato. All the eight known exocyst subunits were identified in *N. benthamiana*, tomato and potato. Similar to Arabidopsis, both tomato and potato contain one copy of *Sec6*, *Sec8* and *Sec10,* two copies of *Sec3*, *Sec5* and *Sec15*, three copies of *Exo84* and multiple copies of *Exo70*, i.e. 22 in tomato and 21 in potato versus 23 in Arabidopsis (Table 1) (Chong et al., 2010). In contrast, *N. benthamiana* has twice the number of exocyst subunit genes (Table 1) which was to be expected based on its allopolyploid genome (Goodin et al., 2008).

Phylogenetic analysis was performed to analyse the evolutionary relationships among the exocyst subunits from *N. benthamiana*, tomato, potato and Arabidopsis. The phylogenetic tree in Figure 1 shows that the Arabidopsis Exo70 proteins cluster together with Solanaceous Exo70s into three clades, i.e. Exo70.1, Exo70.2 and Exo70.3, with Exo70.2 divided over six subclades. The eight subclades named Exo70A–H, have a similar pattern of clade division as previously reported (Chong et al., 2010; Synek et al., 2006). The topological reproducibility of the phylogenetic tree was evaluated by bootstrap analysis. This

phylogenetic analysis of putative Solanaceous Exo70s shows that, similar to Arabidopsis, the Solanaceous plants also have gained numerous *Exo70* genes, which is likely due to ancient gene duplication events during plant evolution.

Table 1. Predicted copy number of exocyst subunit genes in Solanaceous plants.

	Arabidopsis	*N. benthamiana*	Tomato	Potato
Sec3	2	4	2	2
Sec5	2	4	2	2
Sec6	1	2	1	1
Sec8	1	2	1	1
Sec10	1	2	1	1
Sec15	2	4	2	2
Exo70	23	44	22	21
Exo84	3	6	3	3

Arabidopsis Sec3A and Sec3B group together, whereas the Solanaceous Sec3's form two distinct subclades with each containing one member of tomato, one of potato and two of *N. benthamiana* protein members (Figure 2A). Similar to Sec3, Arabidopsis Sec5A and Sec5B share high sequence similarity and cluster together in a comparable manner as observed for Sec3 (Figure 2B). As such it is not possible to distinguish the Solanaceous Sec3A from Sec3B, neither Sec5A from Sec5B. This suggests that duplication of Sec3 and Sec5 occurred twice independently, once in the lineage comprising Arabidopsis and once in the common ancestor of Solanaceous plants.

In contrast to Sec3 and Sec5, the two copies of Arabidopsis Sec15 – i.e. Sec15A and Sec15B – are not grouping together but instead cluster with either Sec15A or Sec15B from Solanaceous plants (Figure 2F). Similarly, the three Arabidopsis Exo84 paralogs were found to cluster with orthologs from Solanaceous plants into three subclades. This indicates that duplication of Sec15 and triplication of Exo84 arose from an ancient duplication in a common ancestor of Brassicaceous and Solanaceous plants (Figure 2G). The phylogenetic trees of the single copy genes *Sec6*, *Sec8* and *Sec10* show clustering of the Solanaceous genes as expected, with the Arabidopsis gene on a separate branch.

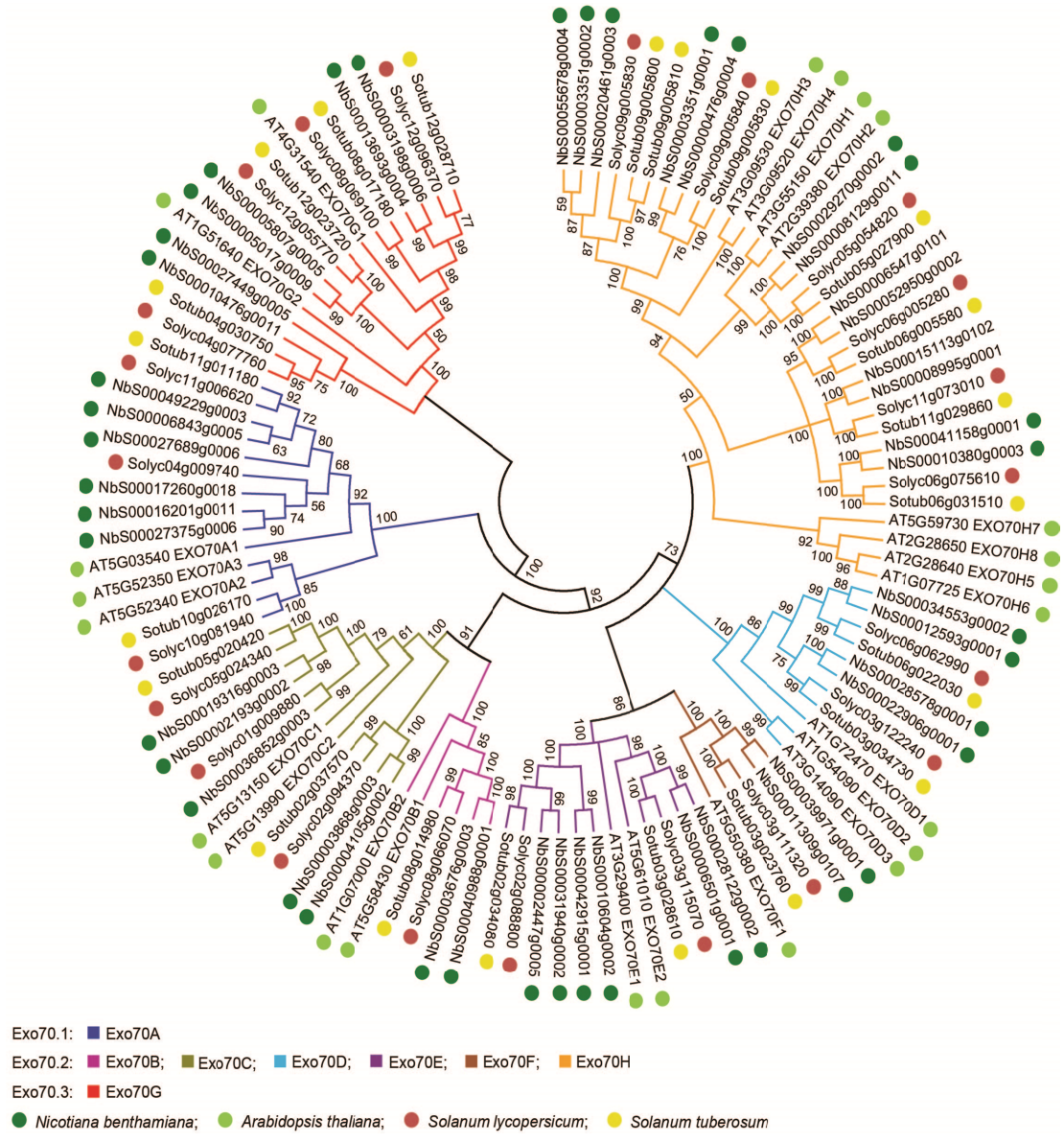


Figure 1. Phylogenetic tree based on predicted Exo70 proteins of Arabidopsis and the Solanaceous plants *Nicotiana benthamiana*, tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*). The Exo70 proteins cluster in 3 clades and 8 subclades distinguishable by the colors of the branches. The colors of the dots correspond to the different plant species. Protein sequences were aligned with ClustalW at default settings and phylogenetic analysis was performed using the Neighbour Joining method with 5000 bootstraps in MEGA 5. Branches corresponding to partitions reproduced in ≤ 50% bootstrap replicates were collapsed.

Our results are in agreement with the findings reported by Cvrckova et al. (2012), who analysed the evolutionary relationship of exocyst subunits from 10 different plant species. They distinguish three groups. The first group includes the low copy number exocyst subunits, i.e. Sec3, Sec5, Sec6, Sec8 and Sec10, all of which are duplicated in one or more plant species likely due relatively recent gene duplication events that occurred independently in different plant lineages. The second group comprises the intermediate copy number subunits Sec15 and Exo84 that increased in copy number much earlier during plant evolution. Similarly, the third group comprising the multi-copy Exo70 family with an enormous diversity among its paralogs is likely generated by ancient gene duplications in the common ancestor of land plants (Cvrckova et al., 2012).

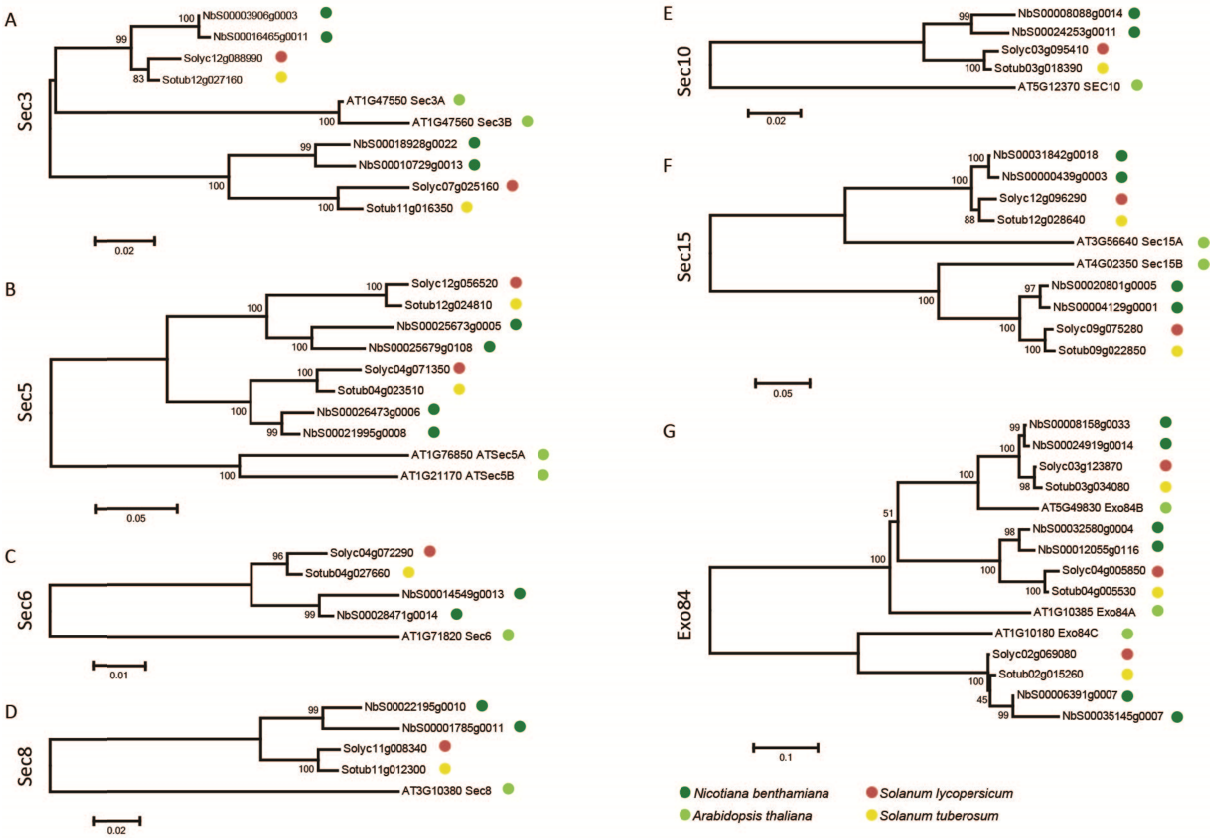


Figure 2. Phylogenetic trees based on the predicted exocyst components Sec3, Sec5, Sec6, Sec8, Sec10, Sec15 and Exo84 from Arabidopsis, *N. benthamiana*, tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*). Protein alignments were constructed using ClustalW at default settings. Phylogenetic analysis was performed by the Neighbour Joining method with 5000 bootstraps in MEGA 5. Branches corresponding to partitions reproduced in ≤ 50% bootstrap replicates were collapsed. Colored dots indicate the different plant species.

Silencing exocyst subunit genes results in a range of developmental phenotypes

In order to silence all exocyst subunit genes in both *N. benthamiana* and tomato, we generated binary Tobacco Rattle Virus (TRV) constructs that are able to target the orthologous genes in the two species. A total 19 constructs was generated, of which 16 have been used for silencing assays in this study. The ones targeting the three *Exo84* paralogs remain to be tested. Sequences of the fragments used for gene silencing are listed in Table S2. Three weeks after TRV treatment, the silencing efficiency was determined by Q-RT-PCR. In all plants tested so far the transcript level of the targeted gene was reduced pointing to efficient silencing in both *N. benthamiana* and tomato (Figure S1). Overall, the gene silencing was found to be more effective in *N. benthamiana* than in tomato (Figure S1). As yet the silencing efficiency of 8 out of the 19 constructs remains to be tested.

Three to four weeks after TRV inoculation, we monitored changes in plant development. Results show that silencing of the exocyst subunit genes with low copy numbers, i.e. *Sec3*, *Sec5*, *Sec6*, *Sec8*, and *Sec10* results in aberrant plant development in *N. benthamiana*. The plants are smaller in comparison to the control, whereas *Sec10*-silenced plants were found to be severely dwarfed (Figure 3). The same holds for some members of the extended *Exo70* gene family namely *Exo70A*, *Exo70C*, *Exo70D* and *Exo70G*. Silencing of other members of this family, i.e. *Exo70B, Exo70E1, Exo70E2, Exo70F* and *Exo70H* and of the two *Sec15* genes, *Sec15A* and *Sec15B* did not cause any clearly visible changes in the growth morphology of the *N. benthamiana* plants. Likely the two *Sec15* paralogous genes are functionally redundant and the same may be true for some of the *Exo70* family members. Another possibility would be that the different paralogs of Exo70 have evolved specialized roles in plant development. Except for silencing of *Sec5* in tomato, which results into smaller plants, silencing of all other exocyst subunit genes did not result to visible changes in plant morphology (Figure S2). This could be due to the lower silencing efficiency in tomato.

Previously Arabidopsis, Exo70A1 was shown to be essential for plant development. Mutation of Exo70A1 resulted in aberrant xylem development and severe dwarfed and sterile plants (Li et al., 2013) whereas, according to Synek et al. (2006), mutations in *Exo70B2*, *Exo70D3*, *Exo70F1*, *Exo70G1* and *Exo70H7*, did not lead to any morphological changes. With the exception of Exo70D and Exo70G, the observations in Arabidopsis are in line with our findings in *N. benthamiana.*

We do find an aberrant phenotype in *N. benthamiana* upon silencing of *Exo70D* and *Exo70G* but this might be due to the fact that instead of targeting just one *Exo70D* gene or just one *Exo70G* gene, as was done in Arabidopsis, we silence all *Exo70D* or *Exo70G* paralogs in *N. benthamiana* simultaneously.



Figure 3. Morphology of *N. benthamiana* plants upon silencing exocyst subunit genes. Pictures were taken three weeks after inoculation with the silencing constructs. Plants inoculated with TRV:*GUS* and TRV:*SGT1* silencing constructs were included as controls.

Exocyst subunits play a role in plant defense against *Phytophthora* pathogens

To investigate whether the individual exocyst subunits are required for defense against *Phytophthora* pathogens, *N. benthamiana* plants in which the various exocyst subunit genes were silenced were inoculated with zoospores of *P. infestans* isolate 14-3-GFP. *Sec10*-silenced plants were not included, since their leaves were found to be too small to use in infection assays. As shown in Figure 4 the leaves silenced for *Sec3*, *Sec6*, *Sec8*, *Sec15A*, *Sec15B*, *Exo70A*, *Exo70B* and

Exo70E2 showed significant larger lesions compared to the control at 6 days after inoculation (dai). Silencing of *Exo70C*, *Exo70E1*, *Exo70F*, *Exo70G* and *Exo70H* did not cause significant differences in lesion areas whereas silencing of *Exo70D* resulted in significant smaller lesion areas suggesting that this subunit plays a negative role in plant defense against *P. infestans*.

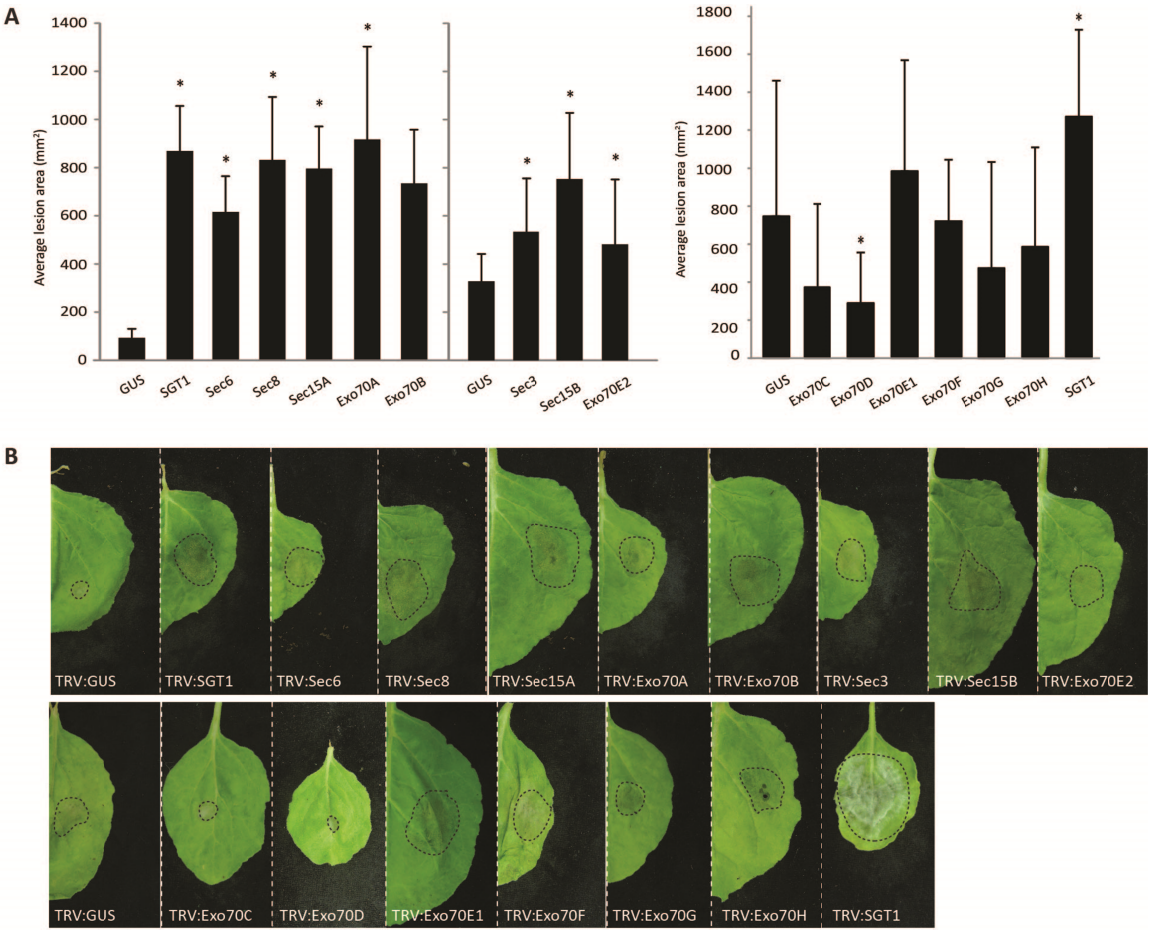


Figure 4. The majority of exocyst subunit gene-silenced *N. benthamiana* plants show enhanced susceptibility to *Phytophthora infestans*.
(A) Average lesion areas 6 dai with *P. infestans* isolate 14-3-GFP. Error bars indicate standard deviation. A significant difference with the control TRV:GUS is indicated by * (N≥12, one-sided Student's t-test, P<0.05). Experiments were performed in multiple batches, and repeated at least twice with comparable outcomes. (B) Lesions caused by *P. infestans* isolate 14-3-GFP on *N. benthamiana* leaves silenced for various exocyst component genes. Pictures were taken 6 dai.

In order to determine whether the exocyst subunits play a role in plant defense against another *Phytophthora* pathogen, we inoculated *N. benthamiana* plants silenced for the individual exocyst subunit genes with *Phytophthora capsici* isolate LT263 using mycelial plugs. No differences in lesion areas were observed 2 dai (Figure S3). However, our results are inconclusive due to the fact that the *N. benthamiana* plants were found to be extremely susceptible to the *P. capsici* isolate used in the infection assays, especially upon inoculation with mycelial plugs as inoculum. This makes it impossible to screen for increased susceptibility. We will repeat these infection assays using zoospores as inoculum or a less aggressive *P. capsici* isolate.

In a similar way we performed infection assays on tomato plants in which the exocyst subunit genes were silenced. Three weeks after TRV inoculation, the fifth and sixth leaf of the tomato plant were used for infection assays with either *P. infestans* or *P. capsici*. Results show that at 6 dai with *P. infestans* all silenced tomato plants had larger lesions compared to the control (Figure 5A and B) and the same was observed on plants inoculated with *P. capsici* at 3 dai (Figure 6A and B).

Taken together, the majority of the exocyst subunits that we have tested seem to be required for defense against *Phytophthora*. From the expanded *Exo70* gene family only members targeted by Exo70A, Exo70B and Exo70E2 TRV silencing constructs seem to be important, but not those targeted by the Exo70C, Exo70E1, Exo70F, Exo70G and Exo70H TRV constructs. One Exo70 subclade, Exo70D, may even play a negative role in plant defense. The *Exo70D*-silenced plants were the only ones in this study that became more resistant to *P. infestans*. In other studies, barley Exo70F and Arabidopsis Exo70H1 were found to be required for penetration resistance against *Blumeria graminis* (Ostertag et al., 2013; Pecenkova et al., 2011) and the latter also for resistance against the bacterial pathogen *Pseudomonas syringae* (Pecenkova et al., 2011). In contrast, in our study we found no indications for a role of Exo70F or Exo70H in *Phytophthora* resistance and this could be due to the fact that different plant pathogens employ different mechanisms for infection. It should be noted though that the silencing efficiency of the TRV:Exo70F and TRV:Exo70H constructs still needs to be determined before we can draw firm conclusions from our infection assays.

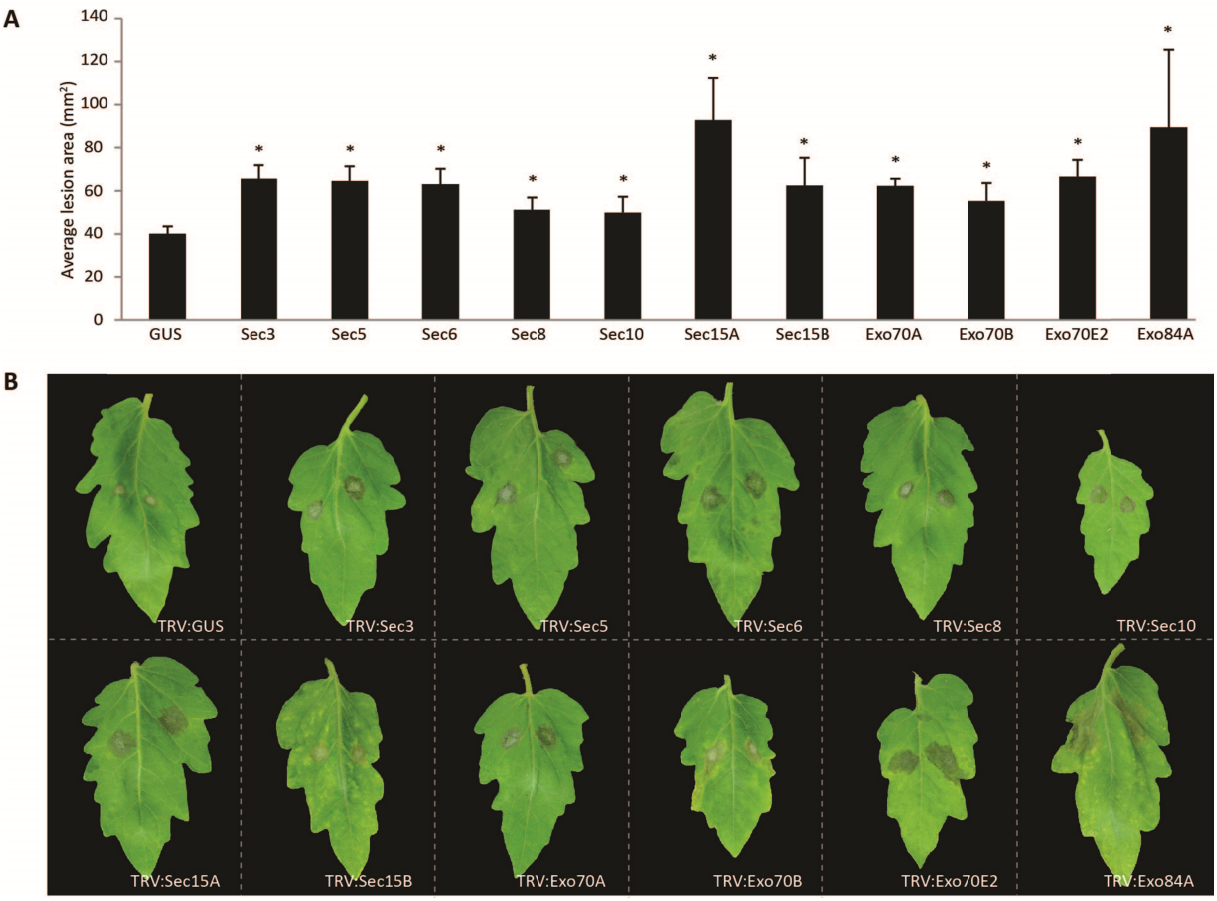


Figure 5. All of the tested exocyst subunit gene-silenced tomato plants show enhanced susceptibility to *P. infestans*.

(A) Average lesion areas caused by *P. infestans* isolate 14-3-GFP on gene-silenced tomato leaves at 6 dai. Error bars indicate standard deviations obtained from at least 22 replicates, with an * indicating a significant difference with the control TRV:GUS (one-sided Student's t-test, $P<0.05$). This experiment was repeated three times with similar outcome. (B) Representative pictures showing lesions caused by *P. infestans* isolate 14-3-GFP on gene-silenced tomato leaves at 6 dai.

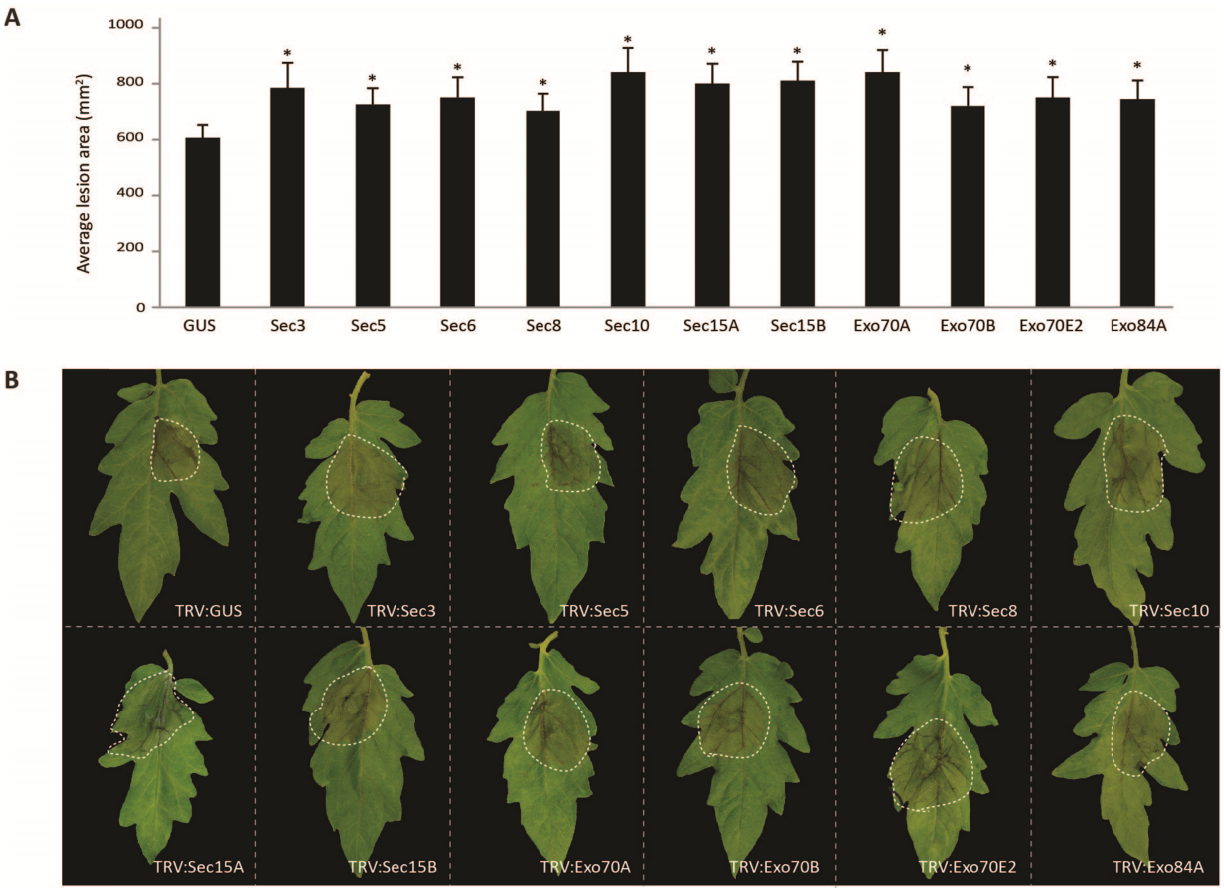


Figure 6. All of the tested exocyst subunit gene-silenced tomato plants show enhanced susceptibility to *Phytophthora capsici*.
(A) Average lesion areas on gene-silenced tomato leaves at 3 dai with mycelial plugs of *P. capsici* isolate LT263. Error bars show standard deviations from at least 22 replicates, with an * indicating a significant difference with the control TRV:GUS (one-sided Student's t-test, $P<0.05$). Experiments were repeated three times with similar results. (B) Representative pictures showing lesions caused by *P. capsici* LT263 on gene-silenced tomato leaves at 3 dai.

Exocyst subunits are involved in callose deposition

Since we previously showed that Sec5 is required for callose deposition (**Chapter 4**), we wondered whether other exocyst subunits are also required for callose deposition. To test this, leaves of silenced *N. benthamiana* plants were infiltrated with *Pseudomonas syringae* pv *tomato* Δ*hrcC* to elicit callose deposition which can be visualized by aniline blue staining. Microscopic analysis of the stained leaves showed significant abundant callose deposition in the TRV:*GUS* plants, while callose deposition in the TRV:*SGT1* plants was severely reduced. In most of the exocyst subunit gene-silenced plants callose deposition was significantly reduced

with the exception of *Exo70A*- and *Exo70D*-silenced *N. benthamiana* (Figure 7A and B). These results indicate that most of the exocyst subunits play a role in callose deposition, possibly because together they form the exocyst that facilitates exocytosis of enzymes that are required for callose synthesis.

Since callose is the major component of papillae, our results are supported by the finding in Arabidopsis that Exo70B2 is required for papillae formation (Pecenkova et al., 2011). The role of exocyst subunits in callose deposition is not surprising, as the exocyst is involved in polarized secretion, which would participate in the transport of callose syntheses to the plasma membrane. In addition, the exocyst may also affect PAMP-triggered immunity (PTI), since the polarized secretion is required for recycling of plasma membrane receptors (Drdova et al., 2013), including the pattern recognition receptors (PRRs) which recognize pathogen PAMPs and mediate PTI.

Sec5 acquired exocyst-independent roles

In **Chapter 4** we have shown that Sec5 is involved in R1-mediated HR, and in **Chapter 5** it was shown that Sec5 is required for cell death triggered by the *P. infestans* elicitors CRN2 and INF1. To test whether other exocyst components also play a role in R1-mediated HR or elicitor-induced cell death, we agroinfiltrated several cell death elicitors and the R1/AVR1 pair into the silenced *N. benthamiana* leaves. Our results show that *Sec5*-silenced plants developed a clear reduction of cell death triggered by CRN2, INF1, and R1/AVR1 at 6 days post infiltration (dpi), which confirmed our previous findings (**Chapters 4 and 5**). In addition, there seems to be a mild reduction of INF1-induced cell death on *Sec3*-, *Sec6*-, *Sec15A*- and *Exo70B*-silenced plants, indicating a possible involvement of the exocyst in secretion of plasma membrane receptors involved in INF1 perception. Except for *Sec6*-silenced plants that show a clear reduction of NLP1-induced cell death, the other plants that we have tested so far show no clear reduction of cell death triggered by R1/AVR1, and neither by CRN2, NLP, NPP1 and BAX (Figure 8). This indicates that the role of Sec5 in cell death development induced by CRN2 or R1/AVR1 is independent of its role in the exocyst complex. Our findings are supported by a study on Arabidopsis Exo70B1, which reported involvement of Exo70B1 in autophagy-related transport (Kulich et al., 2013; Zarsky et al., 2013). This suggests that Exo70B1 acquired exocytosis independent roles. Exocyst components with exocyst-independent roles are known in animals. For example,

the homo-oligomeric Exo70 induces filopodia formation in animal cells, which is independent of its role in exocytosis (Zuo et al., 2006; Zhao et al., 2013). Human Sec5 interacts with and activates the TANK-binding kinase 1 (TBK1) and plays a role in Toll-like receptor-mediated immunity, which is also likely independent of its role in the exocyst complex (Mantovani and Balkwill, 2006; Ishikawa et al., 2009). Together with our findings, we propose that plant exocyst subunits may have exocyst-independent roles. We should note however, that our data are preliminary. They are derived from a single agroinfiltration experiment and need to be repeated.

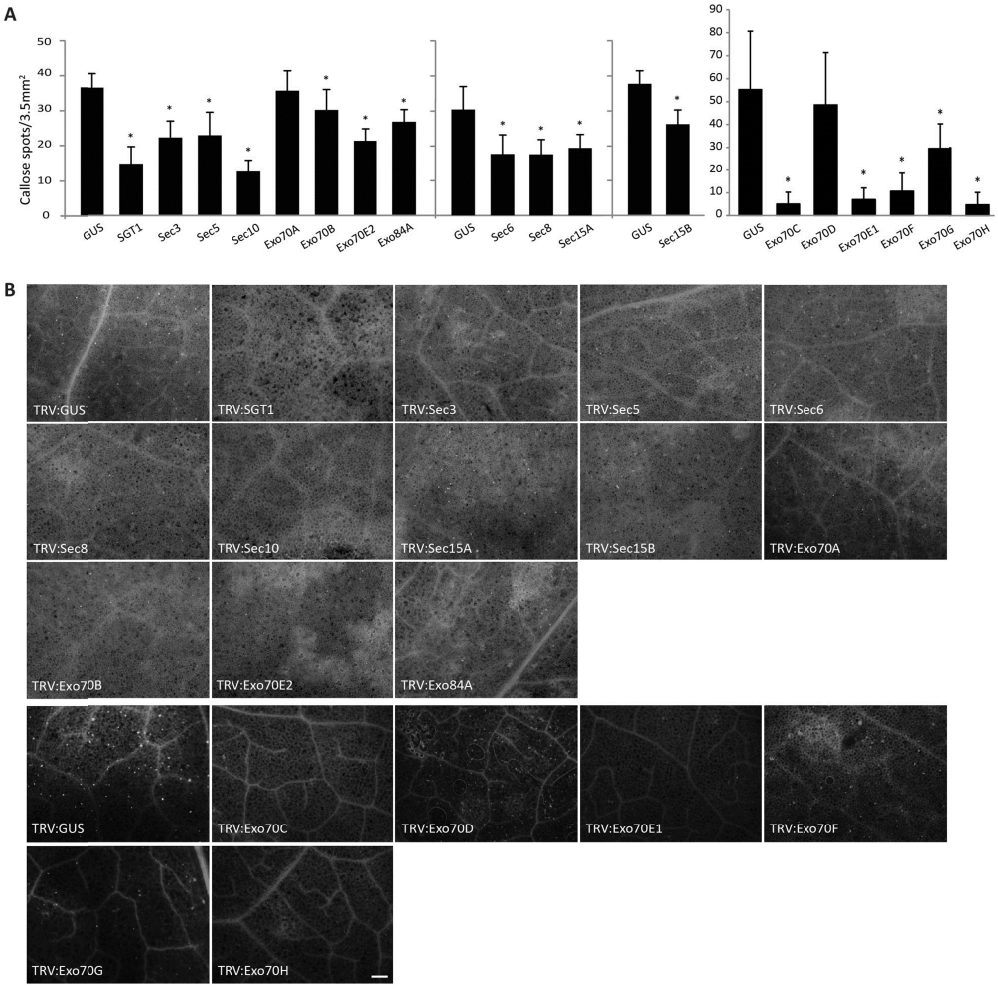
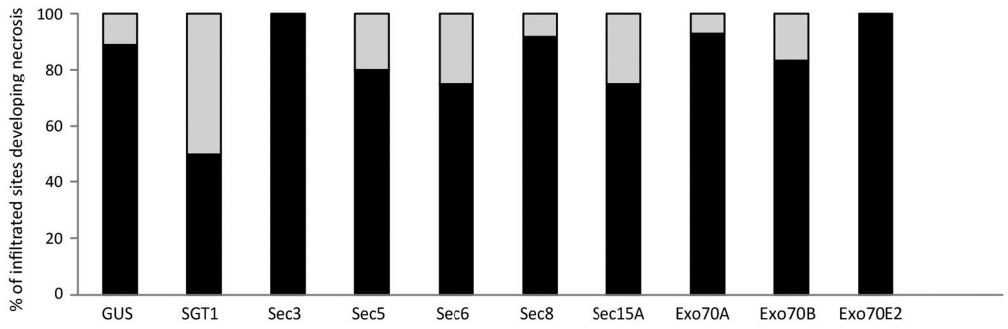
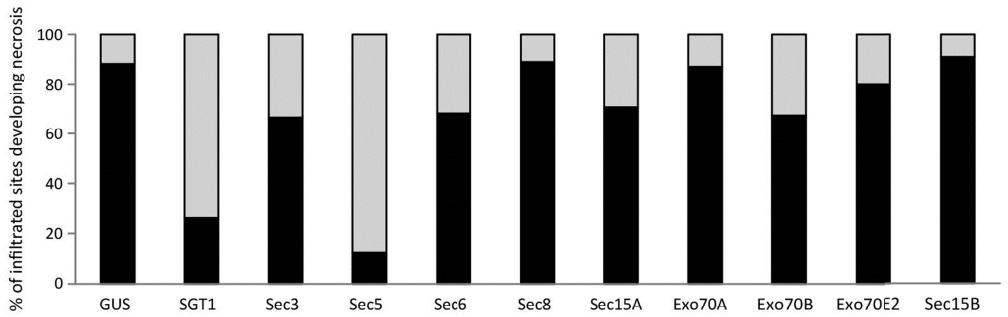


Figure 7. Exocyst subunit genes are required for callose deposition.
(A) Callose deposition in *N. benthamiana* leaves 16 hours after infiltration with *Pst* DC3000 Δ*hrcC.* Y-axis: the amount of callose spots per microscopy picture (3.5 mm^2). Error bars represent standard errors of the mean, and an * marks a significant differences relative to TRV:GUS (N≥9, one-sided Student's t-test, P<0.05). Experiments were performed in multiple batches each containing TRV:*GUS*-treated plants as control. (B) Representative microscopy images showing callose deposition revealed as bright spots upon aniline blue staining. Bar =200 µm.

A



B



C

	BAX	INF1	Crn2	R1/AVR1	NPP1	NLP1
GUS	90	90	100	100	100	100
SGT1	50	30	50	0	100	90
Sec3	100	70	90	100	N.D.	100
Sec5	80	10	30	10	100	40
Sec6	80	70	100	70	100	50
Sec8	90	90	100	80	100	100
Sec15A	80	70	100	90	100	100
Sec15B	N.D.	90	100	N.D.	100	N.D.
Exo70A	90	90	100	90	100	100
Exo70B	80	70	100	90	100	100
Exo70E2	100	80	80	100	N.D.	100

Figure 8. The role of exocyst subunits in cell death development.
(A-B) Graphs showing the percentage of cell death development triggered by BAX (A) and INF1 (B) on different exocyst component gene-silenced *N. benthamiana* plants. TRV:*GUS* and TRV:*SGT1* were used as controls. (C) Percentages of cell death triggered by different cell death elicitors. This experiment was performed once with a total of 10 infiltrated sites per cell death elicitor. Cell death was observed at 6 day post infiltration (dpi).

Conclusion

We have shown that the low copy number exocyst subunits Sec3, Sec5, Sec6, Sec8 and Sec10 are involved in multiple exocytosis-related processes, including proper plant development, defense against *Phytophthora* pathogens and callose deposition, thus indicating that they function in the exocyst complex. For the exocyst in yeast, Sec6, Sec8 and Sec10 are suggested to be the core of the complex (Munson and Novick, 2006). Cvrckova et al. (2012) showed that these three subunits, which are encoded by one or at the most two genes in vascular plants, are the best conserved subunits. This together with our findings suggests that Sec6, Sec8 and Sec10 also form the core of the complex in the plant exocyst. Our studies further show that the Exo70 paralogs play different roles in plant development and defense, and this supports the idea that the Exo70 family has evolved in such a way that each paralog has a specialized role, either in exocytosis or in other cellular processes. In addition to the few studies on the role of the exocyst subunits in development and defense of Arabidopsis, the present study provides insights into the roles of each exocyst subunit in Solanaceous plant development and defense against *Phytophthora* pathogens, and paves the way for further functional investigations of Solanaceous exocyst subunits in defense.

Material and methods

Gene identification and phylogenetic analysis

Protein sequences of Arabidopsis exocyst subunits were retrieved from the TAIR database using the gene inventory of Chong et al. (2009). BLAST analysis was subsequently performed against the genomes and the predicted proteomes of *Nicotiana benthamiana*, *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato) at the Sol Genomics Network (SGN) website (http://solgenomics.net). Protein sequence alignments were constructed using ClustalW with default settings (protein weight matrix GONNET, gap opening of 10, gap extension of 0.2). Obtained sequence alignments were used as input to reconstruct phylogenetic trees using the Neighbour Joining algorithm in MEGA 5 with 5000 bootstrap replicates. Branches corresponding to partitions reproduced in fewer than 50% of bootstrap replicates were collapsed.

Plasmids construction

Multi-sequence alignments of genes encoding *N. benthamiana* and tomato exocyst components were constructed using MultAlin (http://multalin.toulouse.inra.fr) to pinpoint conserved gene segments. Gene segments containing either *Eco*RI/*Sac*I or *Bam*HI/*Sac*I restriction sites were synthesized by Eurofins Genomics (Table S2), and thereafter cloned into the binary vector pTRV2. Binary plasmids were transformed to *Agrobacterium tumefaciens* strain AGL1 by means of electroporation. TRV constructs were generated to silence homologous genes of each exocyst subunit in both *N. benthamiana* and tomato. Gene homologs belonging to clades/subclades *Sec6*, *Sec8*, *Sec10*, *Sec15a*, *Sec15b*, *Exo84A*, *Exo84B* and *Exo84C* were silenced using individual TRV plasmids. Exocyst subunit genes belonging to either clade *Sec3* or *Sec5* were simultaneously silenced using a single TRV plasmid, respectively. *Exo70* gene members were targeted by one single TRV construct per Exo70 subclade, with the exception of *Exo70E* gene members that were silenced using two constructs. The construct used for *Sec5* silencing in tomato and *N. benthamiana* was previously described in **Chapter 4**.

Plant growth and pathogen inoculation

Nicotiana benthamiana and tomato (cv. Moneymaker) were grown in potting soil under standardized greenhouse conditions. *Phytophthora infestans* isolate 14-3-GFP was grown in the dark on rye sucrose agar medium at 18°C, and *Phytophthora capsici* isolate LT263 was cultured on 20% (v/v) V8 juice agar plates (Erwin and Ribeiro, 1996) at 25°C. *P. infestans* zoospores were isolated according to Champouret et al. (2009), where after the inoculum concentration was adjusted to $1*10^5$ zoospores/mL. *N. benthamiana* and tomato leaves were inoculated at the abaxial leaf sides with 10 µl droplets of a *P. infestans* zoospore suspension or *P. capsici* mycelial plugs (Ø 4 mm). Inoculated leaves were placed in trays and incubated at high humidity at 18°C for *P. infestans* or at 25°C for *P. capsici.* Leaves were kept the first 24 hours in the dark, and subsequently at a 16h photoperiod. Lesions diameters were measured at 6 days after *P. infestans* inoculation or 2~3 days after *P. capsici* inoculation. Lesion areas were determined as previously described (Vleeshouwers et al., 1999). *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 *ΔhrcC* was cultured at 28°C in King's B medium and cells were collected and resuspended in 10 mM $MgCl_2$. The inoculum concentration

used for infiltration was set to an OD600 of 0.5 (approximately $1.0*10^8$ cfu/mL) (Kim et al., 2011).

Agroinfiltration and virus-induced gene silencing

Agrobacterium tumefaciens strains containing binary vectors were grown in YEB with appropriate antibiotics at 28 °C. *Agrobacterium* cultures were centrifuged and resuspended in infiltration buffer (per liter: 10 mM MES pH 5.6, 5 g MS salts (w/o vitamins), 20 g sucrose, and 150 µM acetosyringone). Bacterial solutions were diluted to an optical density (OD_{600}) of 1.0 prior to agroinfiltration into 4-week-old *Nicotiana benthamiana* leaves. Co-agroinfiltration of AVR1 and R1 was performed with mixtures of *Agrobacterium* suspensions in a 1:1 volume ratio and a final OD of 0.5.

Agrobacterium strains harbouring pTRV2 derivatives or pTRV1 were mixed in a 1:1 ratio (at a final OD600 of 1.0 for *N. benthamiana* and 1.6 for tomato), and virus-induced gene silencing (VIGS) was performed on 16 day old *N. benthamiana* and 11 day old tomato plants by agroinfiltration into the two first emerging leaves (Peart et al., 2002). TRV:*GUS* and TRV:*SGT1* were included as controls in each experiment (Tameling and Baulcombe, 2007). The fifth and sixth leaf from *N. benthamiana* and the fourth-till-sixth leaf of tomato were harvested three to four weeks after agroinfiltration, and subsequently used for further analysis.

Quantitative RT-PCR

Total RNA was isolated from *N. benthamiana* and tomato leaves by a NucleoSpin RNA plant mini-kit (Clontech). Synthesis of cDNA was performed on 1 µg of total RNA using an oligo (dT) primer and M-MLV reverse transcriptase (Invitrogen). Q-RT-PCR was performed using SYBR Green master mix (Promega), gene-specific primers (Table S1) and 2 µl of 10-times diluted cDNA using a Bio-Rad 7300 thermocycler. Gene expression levels were normalized to actin expression (Gabriels et al., 2007).

Callose deposition assays

N. benthamiana leaf pieces harvested approximately 14 hours after infiltration with *Pst* DC3000 *ΔhrcC* were incubated with ethanol to remove chlorophyll. Cleared leaves were stained with aniline blue and visualized using epifluorescence

microscopy as previously described (Bouwmeester et al., 2011). Four or five leaf disks were examined for every sample, of which three microscope pictures (3.5 mm^2) were taken randomly per leaf disk. Total amounts of callose spots were counted per microscope picture.

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Supplementary information

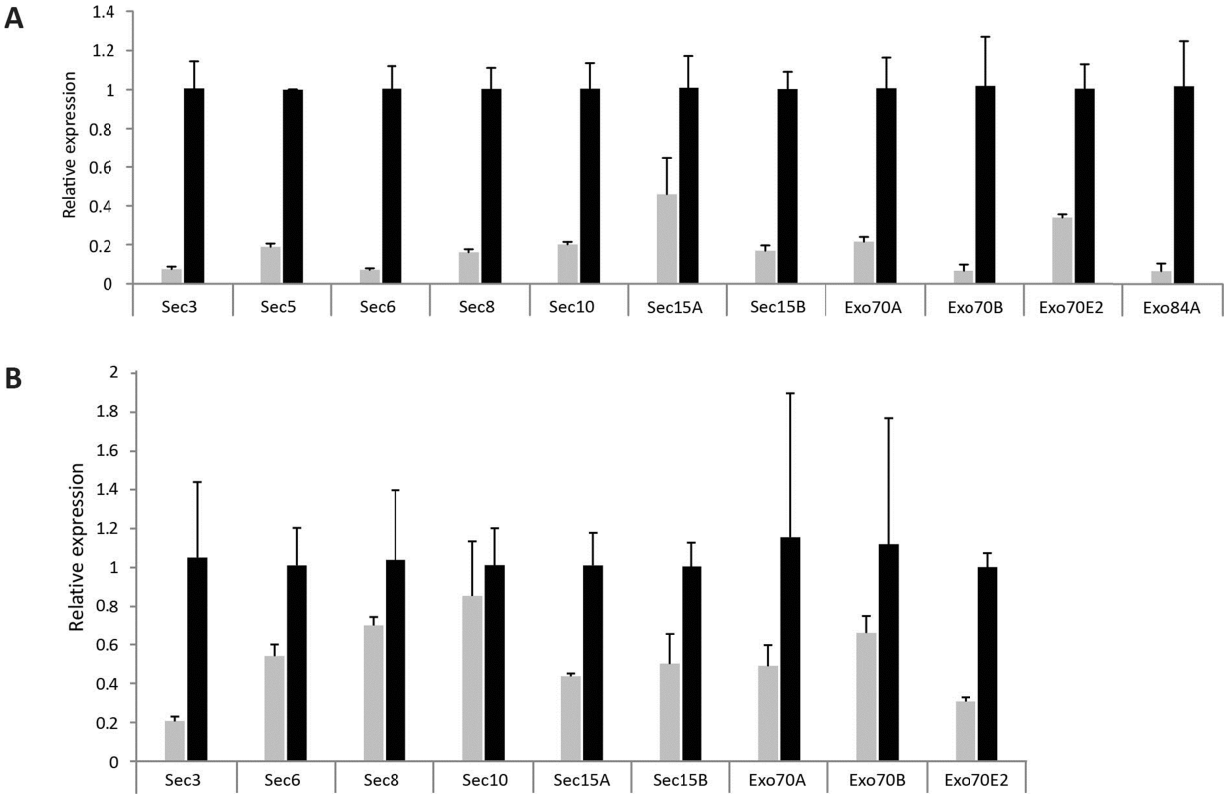

Figure S1. Relative expression of exocyst component genes in silenced *N. benthamiana* and tomato plants.

Relative expression (RQ) of exocyst component genes in (A) *N. benthamiana* and (B) tomato leaves three weeks after TRV treatment. Transcript levels were normalized to Actin, and expressed as mean fold changes (± standard deviation) relative to the transcript level in TRV:GUS treated which was set at 1. (■, TRV:*GUS*; ■, TRV:*Sec5*).

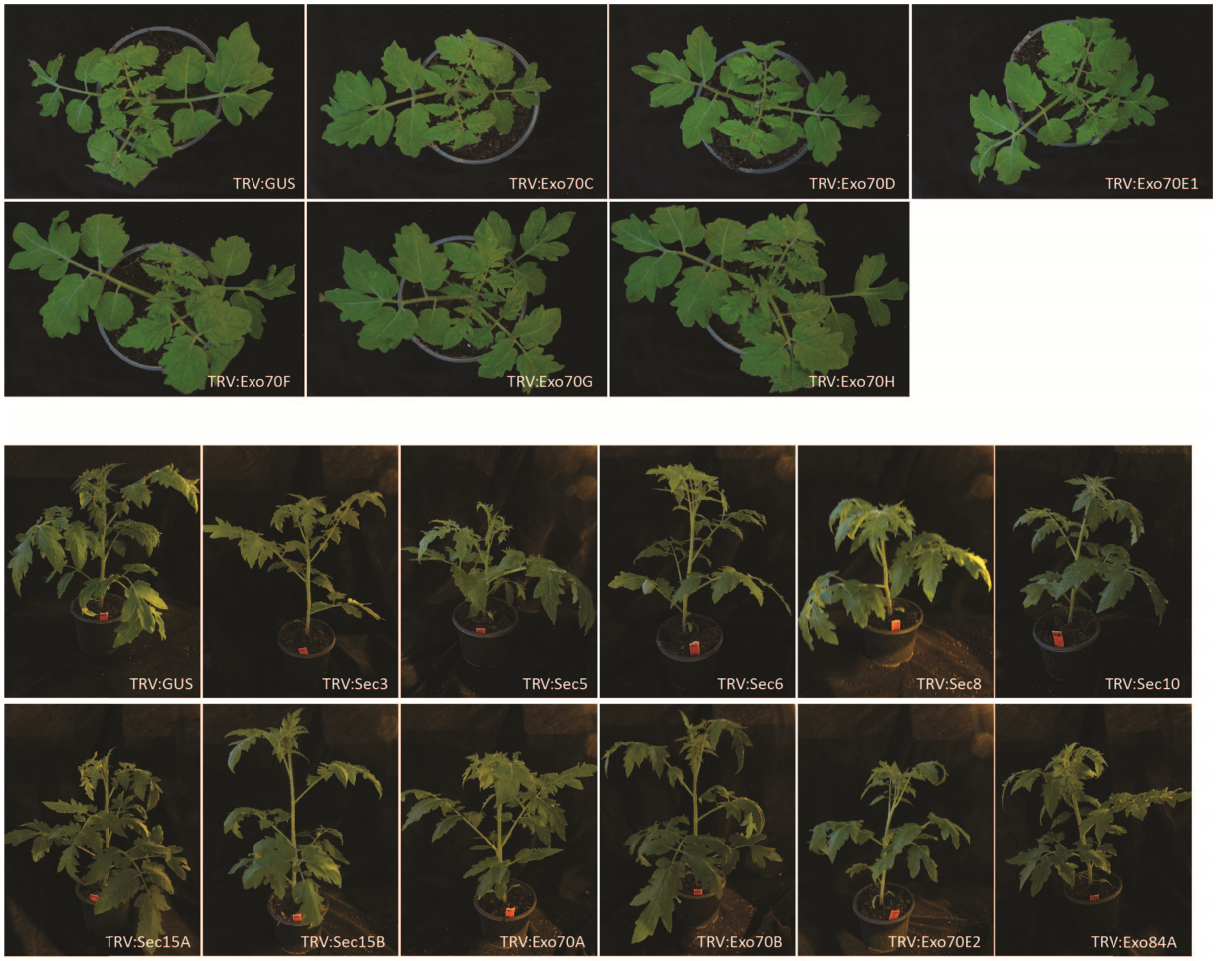

Figure S2. Tomato plants silenced for various exocyst subunit genes.
Phenotypes of different exocyst subunit gene-silenced tomato plants. Pictures were taken 3~4 weeks after TRV inoculation. Upper and lower panels represent gene-silenced plants from two individual batches each containing TRV:GUS-treated plants as control.

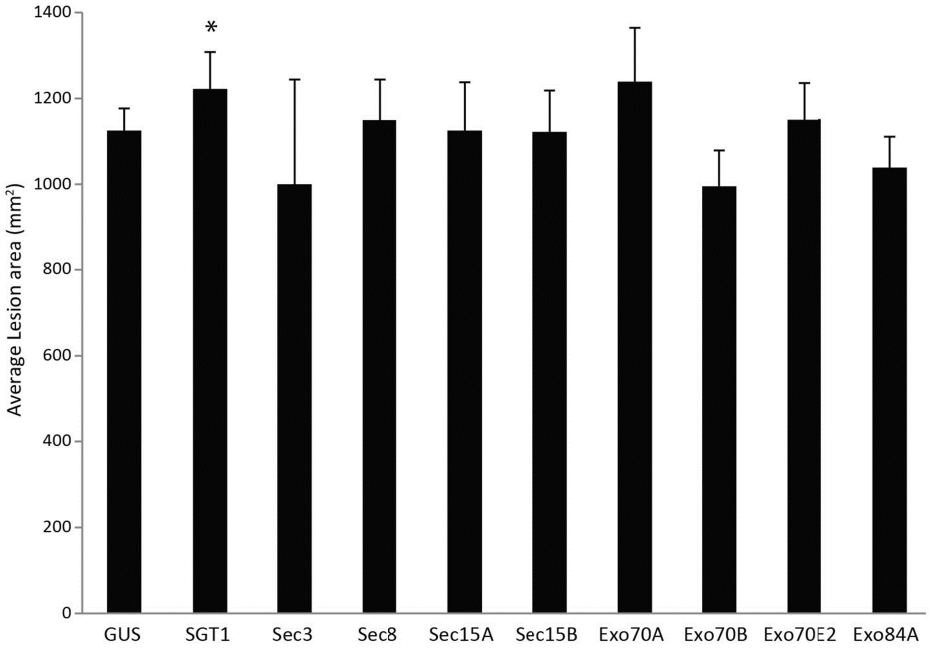

Figure S3. The exocyst subunit gene-silenced *N. benthamiana* plants show no enhanced susceptibility to *P. capsici*.

Average lesion areas 2 dai with mycelial plugs of *P. capsici* isolate LT263. Error bars indicate standard deviation. A significant difference with the control TRV:GUS is indicated by * (N≥6, one-sided Student's t-test, P<0.05). Experiments were performed twice with comparable outcomes.

Table S1. Primers used in this study.

Primer name	Sequence (5'-3')	Primer name	Sequence (5'-3')
Sec3-rtF	agtttgcaaacgagctccgtg	Exo70C1-rtF	tgatgaacaacgggcgatac
Sec3-rtR	actctcatccacaagtagagg	Exo70C1-rtR	attgcaacagtttaccccaag
Sec5-rtf	agttaagacggattgaggaagacc	Exo70C2-rtF	aataccacaagaattaccaaag
Sec5-rtr	tagtggcccaaaagcacgattagc	Exo70C2-rtR	aagtgctctgcgtacgatg
Sec5-F	aaggaattctggtgatgaggattcgtccaag	Exo70D1-rtF	tcccagcttatagatcatttctc
Sec5-R	aacgagctcccaattttagaggatcgacgc	Exo70D1-rtR	ctgagatcttcttcttatgtgttgtg
Sec6-rtF	tcatgaatgcgtatgttgagagaatg	Exo70D2-rtF	catgaactatataagtttaatctcgg
Sec6-rtR	ctcgtacaatttgcacctgct	Exo70D2-rtR	gacaataatccaaatcaaatgaagc
Sec8-rtF	atataccggcctgttcttcag	Exo70E1-rtF	ggccaagctgtagctattgg
Sec8-rtR	tgtacagctttccgataatcaac	Exo70E1-rtR	tgcagaatctccaagatttttca
Sec10-F	aaggaattctgcgtatatctagtgtgggtc	Exo70E2-rtF	aacaggcagccttttgagc
Sec10-R	actggatccaacagcaacttccagccctctac	Exo70E2-rtR	aacatcaaattggcaatgcatc
Sec10-rtF	acaccaccagatgcaggaa	Exo70F-rtF	atgcagctcttgaattttggag
Sec10-rtR	tcttggacagcaacttccttc	Exo70F-rtR	ttctcctaggccatctaacac
Sec15A-rtF	tgctatcaaggctattgatctg	Exo70G1-rtF	accaaagtcttctttgagct
Sec15A-rtR	agtttgcccaatatcctttgc	Exo70G1-rtR	tccagctttccatatttgttc
Sec15B-rtF	tgaagagatgctttcaggtctg	Exo70G2-rtF	aaccattggtacttgtgcaag
Sec15B-rtR	tctcttcagaacttgaacagg	Exo70G2-rtR	ttaactacttgtcgatcaatggc
Exo84A-rtF	tctgaagatcaaaatttctgggaag	Exo70G3-rtF	gaaacgattaaaagcttttaatg
Exo84A-rtR	agcaactgcttcaatagctctgc	Exo70G3-rtR	tccttcttgttcaaccaac
Exo84A-rtF	tgctccttaatgcacattaccag	Exo70H12-rtF	aactatatgcaacaaactatg
Exo84B-rtR	tgtataagctggctctttacc	Exo70H12-rtR	actttgatctcgtctcgtag
Exo84C-rtF	agaatcagctggttgagatc	Exo70H34-rtF	acatcattctacttgctgattac
Exo84C-rtR	agtctcgggataacaaggac	Exo70H34-rtR	agtttgcagagaagaatgagaatc
Exo70A-rtF	agcagttgagaaggatgcaac	Exo70H5-8-rtF1	taactcgatacgtcatgaactacc
Exo70A-rtR	agctgtgaatttgaatctcctcc	Exo70H5-8-rtR1	acaacagaaggcggagagtcc
Exo70B-rtF	tgatgaataatgaaagatacattgttc	Exo70H5-8-rtF2	tgactcggtacgttatgaattatctc
Exo70B-rtR	acttaagcttttctttcaaagacc	Exo70H5-8-rtR2	acggcggaagacggcgagtcat

Table S2. Sequences of the silencing constructs.

Constructs	Sequences
TRV:Sec3	GAATTCCTTCAAAACATTGAGGCATGTGAGTGGTTAACTAATGCTTTACGTGGTCTTGAAGCGCCTAATTTGGATCCTAGCTATGCAAACATGAGAGCTGTAAAGGAGAAACGATCT GAGCTTGATAAGTTAAAAACAACATTTGTTCGAAGAGCATCTGAGTTCTTGAGAAACTATTTTACCAGTTTGGTGGATTTTATGATAAGTGACAAGAGTTATTTTTCTCAGCGTGGAC AATTGAAGAGGCCTGACCATGCTGATCTGAGATACAAGTGCAGGACATATGCTCGTCTTCTGCAGCATTTGAAGAGTC
TRV:Sec5	TTGCTAATCGTGCTTTTGGGCCACTATTTGAAAGACAGGCTCAAGCTGAGAAGATAAGATCTGTTCAAGGAATGCTGCAGAGGTTCCGAACACTATTTAACTTGCCCAGCACGATTCGT GAGAGCATTAGTACTGGTGAATATGACCTAGCAGTCAGGGAGTACAGGAAAGCGAAGTCAATCGTTCTGCCTTCTCATGTTGGAATTTTGAAGCGCGTGCTTGAGGAGGTTGAAAGAG TTATGCAAGAATTTAAAAGCAAGCTTTATAAGTCTCTGGAAGATCCTCAAATTGACCTAACAAATCTTGAAAATAATGTGAGACTCTTGTTGGAGTTGGAACCCGAATCAGATCCTGTGT GGCACTATTTGAATATACAGAACCAGAGAATCCGAGGTTTGCTTGAGAAGTGCACTTTGGATCATGAAGCAAGGACGGAAAATTTCAGGAATGAGATGCGTGAAAGAGCTTTATCTGA TGCAAAATGGAGGCAAATTCAGCAAGATTTGAACCATTCTTCAGATGTTGATTACTCTGATTCTCATGAAAATACATACTTGCCGGGTGATTCACAACAAGTG
TRV:Sec6	TTGACAAGAATTGCTTGGGCCCTTTAAGAAAAGCTTATTGCACATCCCTTAATTTGCTTTTACGCCGGGAGCTCTGGAGCTAAGTTCTAGTACTCTTGAAGCTCTCCCACAGAACTAT GCCGAGCAGGTTAATTTCGAGGACACATGCAAAGGTTTTTTAGAGGTTGCAAAGGAAGCTGTTCATCAAACTGTCAGTGTCATCTTTGAAGATCCAGGAGTAGAGGAATTGCTCGT TAAGTTGTATCAGAAAGATTGGTTGGAAGGACAGGTTACCGAGTTTTTAGTTGCAACATTTTCAGACTACTTCACCGATGTTAAAATGTACATTGAAGAAAGATCATTTAGGCGTTTT GTCGAGGCTTGCCTGGAGGAGACTGTTGT
TRV:Sec8	AGAACCATGTCATTGTTGGAGAACTTCTTGAGTCAAAGTGCTCGCAACAAGTTGACTTGAACACACCTAAGTCCATGCCTACAGACATCAGTTGGAACCCTGATTCAGATGCATCTC GTGACACTGGAGGCTATAGTATTGGCTTTTCATTGACCGTCTTACAGAGTGAATGCCAACAACTCATATGTGAGATCCTGCGAGCTACTCCAGAAGCTGCATCCGCAGATGCTGCTG
TRV:Sec10	GAATTCGCACCGCAAGACACTTTCTGAGCTGGAAAAAGGTGTAGACGGCTTGTTTGATAGCTTTGCACGGTTGGATTTGCGTATATCTAGTGTGGGTCAAACAGCTGCCAAAATAG GAGATCATCTGCAGAGTGCAGATGCTCAGAGAGAAGTTGCTAGTCAGACAATAGAGCTCATAAAGTACTTGATGGAGTTCAATAGCAGTCCAGGAGACCTGATGGAACTTTCTCCA TTGTTTTCTGATGATAGTCGTGTTGCTGAAGCTGCTTCGATTGCTCAAAAATTAAGATCATTTGCTGAGGACGATATTGGAAGGGATCC
TRV:Sec15A	AGATCTAGGTCCTATGGTCAGGCTTTCTTTTGAAAGCGGGAAGCCTGATGCCCTTCTACAACAGCTTAAAAATGTCGTCAAAAAGAAAGAAGTTGAAATAGAGGAGCTTTGTAAGCT TCACTATGAGGAATTTATTGTTGCAGTGGATGAGCTGCGCGGTGTCTTGGTTGATGCAGAAGAGCTAAAAGCTGAGTTGCAAACTGATAATCTGAAGCTGCAAGATGTTGGGAGTA CCCTTCTTTTAAAACTTGAAGAGTTTCTTGAATCTTATTCAATTAAGAAAAATGTTACAGAAGCGATTAAGATGTCAGGCAACTGTGTCCAAGT
TRV:Sec15B	TGCCTGCATTTCCTTATGTTGCACCGTTTTCGTGTACGGTGCCTGATTGCTGTAGAATAGTGCGGTCCTTTATCGAGGATTCAGTAAGTTTTATGTCACATGGTGGTCAGCTTGATTT CTATGATGTGGTGAAGAAGTACTTGGATCGGCTTTTAACTGAAGTCCTGGATGGAGCCCTGTTGAAGCTCATTCATACGTCTATCGGTGGTGTTACCCAAGCAATGCAGATGGCAG CTAACATGGCTGTGTTTGAACGCGCTTGTGATTTTTTCTTTCGTCATGCTGCACAGCTTTCAGGGATTCCATTGA
TRV:Exo84A	ACAGGCAATCCCGTCCTATAGAGCAGAGAGAACTGAAACGGAGACTCCAACGTTTAGTTGATCAGCTCCGTGACAGCTTTTGTAGGCAACATGCACTTGAACTCATTTTCTTGGAA GATGGTGGCGTTCGTTTAAGTCCAGACATGTACCTAAATATGGAAGGAAGTCCAGAAGAGATAGAGTGGTTTCCATCTCCAGTATATCAGGAAATGTTTGAGAAGTTGACTCGAATT GCTAGCATAGCATCAGACATGTTTGTGGGAAGAGAAAGATTTGCTACTATTCTTTTGATGAGACTCACTGAAACTATCA
TRV:Exo84B	AGGCTGGCGATTAGCGAACCAACAACACAATCAAACAAATTTTTATTCTCTTTCTACGAACAACTAATAATCTCCTCTTCTTTCGTCAATCATGGCGATGGCGATAACAACTCTCACC TTAGGATTAGATCTACCCTTTTGATTTTCTTACGTCTTCCCTATTTTTGCTCTATATTTCAACCCTAAACATCCGCCATTAAGATTCACCTCATGGCTTCCGTCAAATCCTCTCGTTCAA GAGCTCATGCGGTCACTCAGTCTAAAGGAATCAACAAGGATACCGGCCCCAAGCTCGAAGAAAATCTTAACGTCTTCAAATCCGACAATTTCGATGCTGATGCTTTTGTCCAGTCCA AATGTCATTCTCTCAACGAGAAGGAAATAAGGCAGCTGTGCTCTTATCTATTGGAACTGAAAAGGGCTTCTGCTGA
TRV:Exo84C	GAATTCTAGTCAAAGAAATAGTGGAGAAGCTCACCCAGTTAGTCATTCTACATTTTGGAGCTAACATATTGACCAGAATTTCACATCTTTTTGACAAGTATGTGGATTCTTTGATTAAA GCCCTGCCTGGTCTTTCTGAGGATGATAATCTCACTGAGCTGAAAGAACCTGTTCTTTTTAGAGCTGAAACAGACTCGGAGCAGCTTGCTCTATTGGGAACTGCATTTACCATTGCT GAGGAACTATTGCCTATGGTTGTATCTAGAATTTGGAATGTTCTTAATGAAAGCAAGGAAGCGAGCTC
TRV:Exo70A	ATTCATTTTATGCGGATTGCTGTCAAATTGTTATTTGCTGCGGAGCGAAAAGTTTGTGATCAAATGTTTGAAGGATTTGAGCACCTCAAAGATCAATGTTTTGCTGAAGTTACTACTG GGAGTGTGGCTGTGCTGCTTAGTTTTGGTGATGCAATTGCAAAAAGCAAAAGATCACCTGAGAAGTTGTTTGTGCTTCTAGATATGTATGAAATAATGCGGGAACTTCATTCAGAGA TTGAATCGCTTTTCATAGGTAAAGCTTGCAATGAAATTAGGGAATCTGCCTTTGGTTTGACA
TRV:Exo70B	TGTTTATGAGGCGCTTAGGGATTTGATGCCTGAATTTGAATTGTTGTTTATGGATCAATACTGTGTGTTATTGAGGAATGAAGCTTTGACTATATGGAGAAGATTAGGTGAGGCTATT AGAGGGATTTTTATGGATTTGGAGAATTTGATTCGCCGTGATCCTGCCAAAACGCCTGTTCCAGGTGGTGGACTCCACCCGATTACTCGATACGTGATGAATTATCTCCGCGCAGCT TGTCGATCCCGGATTTCTCTTGAACAGGTTTTTGAGGAGTCTGCTAGTGTTGTTGATTATAGAGGAGAAGTGGATGATAGAGCGCTTTCATCGTCTCCCTTGGCTGTTCAAATGGCG TGGACCATGGAGTTACTTGAGAGTAATCTGGA
TRV:Exo70C	GAATTCGAGTACAAATATGCTTATTCAATCAGCCGCGTTGATGGTGTTCTTCAACGCGCAATGTCCTGAGAAAATCTTCAGCAATTTGTCTCGAGGTATGATGATCCAACTCCTTAAT TTCGCTGAAGCCGTTTCAAGTGATGGGCGATACGTGGTGTAGGAGACGATCAACGATCGTTAGACATTACCACAAGAATTACCAAAGGGAAACATGGGGTGAAGGTGCTCCAAATCA ATGGGAAAGTGACAAAGCCAGTAGTGAAAGAGAGGTTCAAGAATTTCAGCACAATGCTCGACGAAATCCATCGTACGCAGAGCACTTGGGTGAGCTC
TRV:Exo70D	GAATTCCAGTTATCGATCTACTAACAGCATTCGTGAAGTCGATCTCATGCCGTCAGAAGCAATATACGATCTCCGGTGCATCGCGGAGCGAATGATTTCAGCTGGATATCTCCGGG AGTGTATTCAGGTGTATGGCAGTGTACGCAAGTCTGCTGTGGATTCGAGCTTCCGGAAGCTTGGAATAGAGAAGCTGAGCATAGGAGATATACAGAGACTTGACTGGGAGACTCTC GAAGCAAAGATTCGTAGATGGATACGAGCAGCAAAAGTATGCCTTCGCATACTTTTCGCTAGCGAGAAGAAGCTCTGTGAGCAAATGAGCTC
TRV:Exo70E1	GAATTCTGATGAACAACATCCATTACATGGTTCAGAAAATCAAGAACTCCAAAATTAGAACTTATTTTGGTGATGAGTGGATAAAAACACATATTGTAAAATACGTGCAGCACGAAAA AAGCTACGAGAGAATAACATGGAGTTCAATCATATCTTTGATAACCGGTTATGAGAAGTTAGGGAAGGCAGTTCTGAAGGAGAGGTGCAGAAATTTCAGTATTGCTTTTGAGGAAGT GTATAAAAACCAGACAGGATGGACTATTCCAGATATTGAGAGCTC
TRV:Exo70E2	GCATTTATCAATGTCAGAAAAGATGCCTTGGACGATTGCCTCTTCATTCTTGAAGTTGAGAGGTTGAGCATTGAGGATGTACTGAAGATGGAATGGAACTCGTTGAACTCCAAAATCAG GAGGTGGATACGGGCTATGAAGATCTTTGTGCGCGTTTATCTTGCCAGTGAAAAGTGGCTAAGTGATCAGATTTTTAGCGAGCTGGAAGCAGTTAGTTCAGTTTGCTTTGCTGAGGCC TCAAAAGCTTCAATCGTACAGCTTCTGAACTTTGGTGAAGCCATAGCTATTGGCCCTCATCAACCGGAGAAGTTGATTCGGATTCTTGACATGTATGAGCTGCTCGCAGATCTTATCCC AGATATTGATGCTATGTACTCTGA
TRV:Exo70F	GAATTCATTGATCTGAGAGAGATTGCTAACCGTATGATTCGATCTGGATATGAAAAGGAATGTTGTCAAGTGTACTCTAGTGTTCGTAGAGAGGTGCTTGATGAGTGTTTAGCAATTC TTGGCATTGAGAAACTGAGCATTGAGGAGGTCCACAGGATTGAATGGCAGTCGCTGGACGAGAAAATGAAGAAATGGATTTATGCTGTAAAAGTTCTGGTGAGAATCCTTTTATCTG CTGAAAAGAGCTTGTGTGAACAGATTTTTGTAGGTTCTGAGTTGATTAAAGAAGTGTGCTTTATGGAAACTGCAAAGGGCTGTGTGAGCTC
TRV:Exo70G	GAATTCGACAAGATAATGGCTGTCTTCTTTAGATTCGGGGAAGGGGTTGCACGAAGTAAGAAAGAACCCCAAAAGCTGTTCAAGCTGTTAGATATGTTTGAATCATTGGAAAGGCTA AAATGGTAGTGTTCCGAGGCTGGTTAACTTTGTCAGTGAGTATTGTAATCAGATTCTTGAGGATGAATATTGGTCCACACTTATCCAAGTTGTGGAGATACATCAAAGTTTGCAGGCA CTTAATTTGGATTACCTTGAGATTTCAGTCTCCGAGTTCAATGACGTGCAGAGCATAGAGGGTCACATTGCTCAGTGGGGTAAGCATTTAGAATTTGCTGTAAAGAGCTC
TRV:Exo70H	GAATTCAAACTTGATGCAAAAGGCTATGAAAAGACTTGAAAATGAATTTTACCAAATTTTGTCAACAAACAAGGAGCATTTAGATCCAGAATCAGTATCTAAGTCTAGTCGTTAGGTG AAAATTGGATATCAAAACAGGAGGGAAAAATCAAGCAGTTTGCATCAAACTATGAACGGCTAGGATGGAGCCACGTCATTGAATCTCCCTTTTATGTAAACTCGATGGTAAAGCACA ACTTTACAAGGACGTTTCATTATCCTATCTGTTCTTAGCGAACAACTTAAACTATGTCGTTTCAGAGCTC

Chapter 7

General discussion

General discussion

In response to pathogen attack, host plants have evolved multi-layered defense responses (Jones and Dangl, 2006). Basal defense, which is generally believed to function as the first layer of plant defense, is mediated by plasma membrane localized pattern recognition receptors (PRRs). These PRRs detect pathogen- (or microbe-) associated molecular patterns (PAMPs or MAMPs), and initiate PAMP (MAMP)-triggered immunity (PTI/MTI). To avoid PTI, plant pathogens secrete proteins named effectors that manipulate host basal defense. In response, host plants activate the second layer of defense response, which in most cases is mediated by intracellular receptors that belong to the group of nucleotide-binding and leucine-rich repeat domain containing (NLRs) proteins. Pathogen effectors are recognized by corresponding NLRs, and this subsequently leads to effector-triggered immunity (ETI) that stops pathogen colonization. ETI is often coupled to host programmed cell death, also known as hypersensitive response (HR). However, plant pathogens can avoid ETI by altering their effector repertoire via natural selection and this makes breeding of durable resistant cultivars a challenging task. Consequently, understanding the mechanisms of effector-modulated and effector-triggered host defense is essential to develop sustainable methods for plant disease control.

This Chapter focuses on how pathogen effectors on the one hand, manipulate host defense and on the other hand, trigger host immunity mediated by corresponding host resistance proteins. In this thesis, we particularly focussed on the *P. infestans* RXLR effector AVR1 and studied the mechanisms of AVR1-triggered R1-mediated immunity and AVR1-triggered host susceptibility. Here, I will discuss our findings from a general perspective, and with an emphasis on the current developments in the field of effector biology.

Effector identification

Plant pathogen effectors are small secreted proteins that modulate host cellular processes (Hogenhout et al., 2009). Bacterial pathogen effectors were identified during studies on the type III secretion system (T3SS) (Abramovitch et al., 2006; Block et al., 2008). T3SS-secreted proteins were firstly identified as avirulence effectors based on their avirulence activities on plants harbouring corresponding NLR proteins. Subsequently, they were also found to contribute to virulence on plants lacking corresponding NLR proteins. Due to the development of genome sequencing and the availability of bacterial genome data, an increasing number of

bacterial effectors has been identified by different methods. For identifying conserved effectors, homology search methods are often used (Alto et al., 2006; Arnold et al., 2009; Studholme et al., 2009). Novel effectors have been identified via sequence-based methods to extract discriminant sequence features (Wang et al., 2011; Ferreira et al., 2006; Yang, 2012; Wang et al., 2014). In addition, domain-based methods were applied to pinpoint novel effectors, including prediction of conserved domains and protein secondary structures (Panina et al., 2005; Sato et al., 2011).

The first effectors of filamentous plant pathogens were identified based on their avirulence functions. For example, the first identified fungal avirulence effector *Cladosporium fulvum* AVR9 was purified from apoplastic fluid based on its ability to elicit a hypersensitive response when infiltrated in tomato plants carrying the matching *Cf9* resistance gene (van Kan et al., 1991). *Phytophthora sojae* AVR1b was the first oomycete avirulence gene cloned and this was done by map-based cloning (Shan et al., 2004). Subsequently, more fungal avirulence effectors were identified through these methods of map-based cloning and analysis of the secretome during host infection (Stergiopoulos and de Wit, 2009; Mesarich et al., 2014). Although the availability of whole genome sequences has provided an opportunity to predict putative effectors by searching secreted small cysteine-rich proteins, fungal effectors are in general difficult to predict due to lack of conserved motifs (Catanzariti et al., 2007). As a result, other methods such as comparative secretome analyses, transcriptional profiling, or genome-wide associate studies, are needed to narrow down candidate effectors before going for functional analysis.

In contrast to fungal pathogen effectors, conserved motifs have been identified in oomycete pathogen effectors. The most outstanding example is the conserved RXLR motif which was initially identified in a small diverse set of avirulence effectors cloned from oomycete plant pathogens, including *Hyaloperonospora arabidopsidis*, *Phytophthora infestans*, and *P. sojae*. They were found to share a conserved RXLR (arginine, any amino acid, leucine and arginine) motif at their N-termini (Rehmany et al., 2005), that was later shown to be needed for host translocation (Rehmany et al., 2005; Whisson et al., 2007). Another example is the identification of a conserved N-terminal LXLFLAL motif in Crinklers (CRNs), that also seems to be required for host translocation (Schornack et al., 2010). The available oomycete genome sequences facilitated the prediction of hundreds of RXLR effectors and CRNs (Stassen and van den Ackerveken, 2011).

Filamentous pathogen effectors can roughly be divided into two groups, one group comprising effectors that function in the host apoplast and known as apoplastic effectors, and the other group comprising effectors that are translocated into the host cell and function intracellularly, the so-called intracellular effectors. Although several RXLR effectors and CRNs were shown to function inside the host cell (Whisson et al., 2007; van Damme et al., 2012), it cannot be ruled out that some of them may also function in the apoplast. For example PEX147, which later turned out to be the intracellular *P. infestans* RXLR effector AVR3a, was initially identified in culture filtrate of *P. infestans* grown in a synthetic medium (Torto et al., 2003; Armstrong et al., 2005). In addition, a recent study on the extracellular proteome of *P. infestans* showed that multiple RXLR effectors and CRNs are present in the culture filtrate from synthetic medium (Meijer et al., 2014). These findings indicate that RXLR effectors and CRNs are not solely secreted to interfere with intracellular host targets, they may also have targets in the extracellular environment. This is in line with the finding that the *P. infestans* RXLR effector IPI-O1 interacts with the extracellular domain of the lectin receptor kinase LecRK-I.9 via a RGD motif and in this way disrupts host plasma membrane-cell wall adhesions likely to facilitate infection (Bouwmeester et al., 2011a). Taken together, no clear cut between intracellular and extracellular effectors can be made.

Effector translocation

Avirulence effectors from filamentous plant pathogens are predicted to translocate into the host cell and this is in line with the fact that for many of them their corresponding resistance proteins localize inside the host cell. Oomycete pathogen effectors were found to contain the conserved N-terminal motif RXLR, which was shown to function in host translocation. Mutation of the RXLR motif from *P. infestans* AVR3a abolished the recognition of AVR3a by its corresponding R protein R3a, suggesting that translocation to the host cytoplasm was hampered (Whisson et al., 2007). Later, similar studies showed that the RXLR motif is able to translocate effector proteins into the host cell independent of the pathogen (Dou et al., 2008; Bhattacharjee et al., 2006). These findings however are still under debate (Bouwmeester et al., 2011b; Petre and Kamoun, 2014). Fungal pathogen effectors are highly divergent in their sequences even within a single species, and so far no clear conserved motif in fungal pathogen effectors has been identified that may function in translocation to the host cell (Rafiqi et al., 2012). For

example, although it is known that the translocation of the rust fungi effectors AvrM and AvrL567 into the host cell is dependent on the N-terminal region, there were no conserved sequence features found in this uptake signal region (Rafiqi et al., 2010).

Effector subcellular localization

Live-cell imaging enables us to investigate the localization of effectors *in planta*. In terms of localization the best studied fungal effectors thus far are from *Magnaporthe oryzae*. By using transgenic *M. oryzae* strains secreting fluorescently labelled effectors it was shown that these effectors are able to translocate into a host cell and in this way, the dynamics of effectors inside host cells could be monitored. *M. oryzae* secretes its effectors into a host-derived structure, called the biotrophic interfacial complex (BIC), which is only observed during successful host infection (Mosquera et al., 2009). A recent study further showed that *M. oryzae* develops a Golgi-independent secretion system for effector focal secretion into BICs (Giraldo et al., 2013).

The subcellular localization of various RXLR effectors has been studied by transient expression of fluorescently tagged effector proteins in plant cells and this showed that RXLR effectors localize to various subcellular compartments. For example, of the 49 RXLR effectors of the downy mildew *H. arabidopsidis* (Caillaud et al., 2012) that were investigated for their *in planta* subcellular localization, 32 (66%) were localized in the plant nucleus, half of which were also found in the cytoplasm. Of the remaining 17, 13 localized on the plasma membrane, 3 in the cytoplasm and 1 in vacuoles. Subcellular localization of several *Phytophthora* RXLR effectors was investigated together with their biological functions. For example, *P. sojae* Avh241 and *P. infestans* AVRblb2 both localize on the plasma membrane. The membrane localization of Avh241 was found to be critical to induce plant cell death (Yu et al., 2012). AVRblb2 was shown to accumulate around the haustoria upon infection likely to inhibit the secretion of a host cysteine protease C14, which was identified as a host target of AVRblb2 (Bozkurt et al., 2011). RXLR effectors can localize to different cellular compartments, e.g. *P. infestans* AVR2 localizes in the nucleus and cytosol as well as the plasma membrane, while its membrane localization is required to trigger a R2-mediated hypersensitive response (HR) (Saunders et al., 2012). The *P. infestans* RXLR effectors PexRD2 and AVR3a localize in the cytoplasm, where PexRD2 was shown to bind to MAPKKKε (King et al., 2014) and AVR3a to stabilize a host E3 ligase

CMPG1, both to hamper host immunity (Bos et al., 2010). RXLR effectors can also localize to the host ER membrane. This was shown to be the case for *P. infestans* RXLR effector Pi03192 that interacts with and prevents relocalization of two plant NAC transcription factors from the ER to the nucleus (McLellan et al., 2013).

We have analysed the localization of the *P. infestans* RXLR effectors AVR1 and AVR1-like by expressing GFP-tagged AVR1 and AVR1-like (A-L) *in planta* and shown that both localize to the nucleus and cytoplasm of the plant cell (**Chapter 3**). To investigate the biological relevance of this nucleocytoplasmic partitioning of AVR1, we modified the localization of AVR1 to either the nucleus or cytoplasm by fusing a nuclear localization signal (NLS), or a nuclear export signal (NES) to AVR1 (**Chapter 3**). When targeting AVR1 to the nucleus ($AVR1^{NLS}$) it is able to trigger R1-mediated HR but in contrast, when targeted to the cytoplasm ($AVR1^{NES}$) it largely loses this ability. This shows that AVR1 has to be in the nucleus to trigger HR mediated by R1. A way for *P. infestans* to circumvent recognition by R1 could be to avoid the nuclear localization of AVR1. However, this is not the case, as the unrecognized AVR1-like is still able to localize to the nucleus without being recognized by R1. We hypothesize that AVR1 may have essential nuclear functions. There are two observations that support this hypothesis. First, $AVR1^{NES}$ is toxic to the host cell; overexpression triggers host cell death but in the presence of $AVR1^{NLS}$ this $AVR1^{NES}$ induced cell death is suppressed completely. Second, in a Y2H screening we found several putative nuclear-localized host proteins as candidate interactors of AVR1, including a TGA motif-binding bZIP transcription factor. This suggests that AVR1 may also manipulate host transcription in order to modulate immunity. These observations indicate that AVR1 likely targets different host proteins in different host subcellular compartments to manipulate host immunity. For example, the cytoplasmic localized AVR1 targets the exocyst component Sec5 to interfere with host immune secretion (**Chapter 4**), while the nuclear localized AVR1 may target host transcription to suppress host immune transcription. Further experiments are needed to characterize these putative host targets of AVR1 and to better understand the mechanisms underlying AVR1 triggered host susceptibility.

We have shown that, similar to AVR1, R1 localizes to both the nucleus and the cytoplasm (**Chapter 3**). Artificially targeting of R1 solely to the nucleus did not alter R1-mediated HR and resistance whereas targeting R1 to the cytoplasm completely abolished its function. These results indicate that R1 functions inside the host nucleus. In this respect R1 behaves similar as several other R proteins

such as barley MLA10, Arabidopsis RPS4 and SNC1, which have also been shown to function inside the nucleus (Shen et al., 2007; Wirthmueller et al., 2007; Cheng et al., 2009). Taken together, we hypothesize that R1 possibly perceives AVR1 in the cytoplasm and subsequently activates immune responses inside the host nucleus.

Effector-triggered host immunity

Although RXLR effectors have conserved N-terminal domains, their C-terminal effector domains are highly diverse and show no sequence similarity with any known proteins. Jiang et al, (2008) compared sequence features of all the RXLR effectors from *P. sojae* and *Phytophthora ramorum* and identified three conserved motifs in the C-terminal effector domains known as W, Y and L motifs. These motifs are present in more than half of the investigated RXLR effectors and range in length from 21 to 30 amino acid residues. Multiple studies have shown that the C-terminal domains with these conserved motifs are sufficient to trigger HR mediated by the corresponding R protein. For instance, a 34 amino acid region in the C-terminus of *P. infestans* AVRblb2 is sufficient to trigger Rpi-blb2-mediated HR (Oh et al., 2009). Similarly the C-terminal region of AVRblb1 (IPI-O1) that contains a W motif is able to trigger Rpi-blb1-mediated HR (Champouret et al., 2009). *P. infestans* AVR4 contains three W motifs in its C-terminus, and the combination of W2 with either W1 or W3 is sufficient for triggering R4-mediated HR (van Poppel et al., 2009). *P. infestans* AVR3a needs the 75 amino acid C-terminal region to trigger R3a-mediated HR (Bos et al., 2006). The *P. sojae* RXLR effector AVR4/6 is the only exception known so far where the N-terminal region contains determinants for recognition. This RXLR effector is recognized by two different host NLR proteins, i.e. RPS4 and RPS6. RPS6 was shown to recognize the C-terminus of AVR4/6, while RPS4 is recognizing the N-terminal region (Dou et al., 2010).

AVR1 as well as AVR1-like contain three conserved motifs in the C-termini, two W motifs and one Y motif (**Chapter 2**), and share 83% amino acid similarity in the region comprising the three motifs. Compared to AVR1-like, AVR1 has a C-terminal 38 amino acid extension (here named T-region), which shares no sequence similarity with any known protein. *P. infestans* isolates that have the *AVR1* gene are recognized by potato cultivars carrying the NLR resistance gene *R1* and this triggers a HR that arrests pathogen growth. HR is also observed when AVR1 is co-agroinfiltrated with R1 into *N. benthamiana* leaves. We have found

that deletion of the T-region in AVR1 does not completely abolish HR, but instead it leads to a delay of HR development (**Chapter 2**). Also, the T-region alone is not sufficient to trigger R1-mediated HR. However, it is important for R1-medited HR, since it is able to activate R1-recognition of AVR1-like when it is fused to AVR1-like or transiently co-expressed with AVR1-like and R1 into the *N. benthamiana* leaves. The role of the T-region in R1-mediated HR is further demonstrated by the fact that when the T-region is deleted, all three motifs in AVR1 are required for triggering HR. Altogether, our results indicate that R1 is recognizing both the conserved C-terminal motifs (i.e. W1, W2 and Y) and the T-region of AVR1. AVR1-like seems to overcome R1 recognition not only because it lacks the T-region but also because it has different epitopes in the three conserved motifs.

Effector-mediated host susceptibility

To promote colonization, filamentous plant pathogens secrete hundreds of effectors during infection to target different layers of plant defense responses (**Table 1** and **Figure 1**). Plant pathogens have different mechanisms to establish infection. Necrotropic plant pathogens extract nutrients from dead plant tissue, and thus secrete various toxic compounds and cell wall degrading enzymes to induce host cell necrosis. Moreover, necrotropic plant pathogens are able to make use of plant defense responses to facilitate colonization. For example, the fungus *Cochliobolus victoria* secretes the toxin victorin to target the defense-associated thioredoxin TRX-h5, which is guarded by the Arabidopsis NLR protein LOV1, and thus activates LOV1-mediated cell death (Lorang et al., 2012). In contrast, biotrophic and hemibiotrophic plant pathogens require living host cells for proliferation. As a result, they have evolved different strategies to suppress the accumulation of toxic proteins or secondary metabolites, or to interfere with programmed cell death induced by host immune responses.

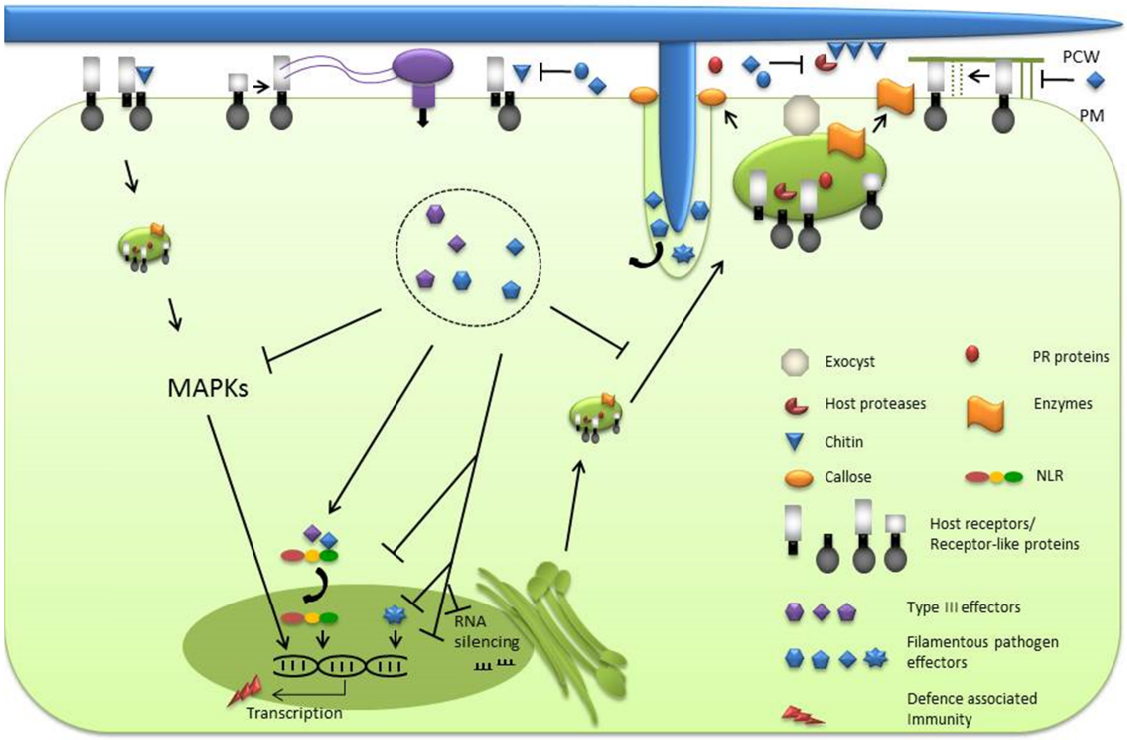

Figure 1. Plant pathogens effectors target various host cellular processes. Host plants have evolved different immune receptors to perceive pathogen effectors and trigger immunity. Pathogen effectors can either be targeted to the apoplast or the host cell and manipulate immunity in different ways.

The apoplastic effectors of filamentous plant pathogens employ in general three strategies to manipulate host immunity, (i) by inhibiting host proteases (Ökmen and Doehlemann, 2014), (ii) by targeting fungal cell walls to protect their hyphae or to inhibit chitin-induced host immunity (Rovenich et al., 2014), and (iii) by detoxification of plant secondary metabolites (Ökmen et al., 2013). The intracellular effectors from filamentous plant pathogens have evolved diverse ways to suppress plant defense responses. The RXLR effectors PSR1/PSR2 from the oomycete plant pathogen *P. sojae*, target and suppress the host RNA silencing pathway to facilitate pathogen colonization (Qiao et al., 2013). The oomycete pathogen *Phytophthora parasitica* secretes the RXLR effector PSE1 during penetration to control the auxin content (Evangelisti et al., 2013). The maize smut fungus *Ustilago maydis* secretes the Tin2 effector to stabilize the active protein kinase ZmTTK1 that functions in anthocyanin biosynthesis, likely to suppress lignification (Tanaka et al., 2014). RXLR effector Pi03192 from *P. infestans* perturbs host transcription by targeting and preventing re-localization of host NAC transcription factors to the nucleus (McLellan et al., 2013). *P. sojae* RXLR effector AVR3b is an ADP-ribose/NADH pyrophosphorylase, which suppresses ROS accumulation at the infection site (Dong et al., 2011).

P. infestans RXLR effector AVR3a and the *Magnaporthe oryzae* effector AVRPiz-t are able to target the host ubiquitination system to suppress PTI (Bos et al., 2010; Park et al., 2012). *P. infestans* PexRD2 and *P. sojae* Avh331 are reported to target the host MAPK signalling pathway. AVRblb1 (IPI-O1) targets the plasma membrane lectin receptor kinase LecRK-I.9 to disrupt plasma membrane-cell wall adhesions (Bouwmeester et al., 2011a), whereas *P. infestans* RXLR effector IPI-O4, an AVRblb1 variant is able to suppress IPI-O1 to avoid Rpiblb1-mediated resistance (Chen et al., 2012). Several other RXLR effectors are able to suppress plant PTI responses but as yet the mechanisms are unknown (**Table 1**).

Table 1. Pathogenic fungal and oomycete effectors impede different layers of plant immunity.

Effector	Species	Target	Processes affected	References
BFA	*Alternaria carthami*	unknown	inhibits host vesicle trafficking	Driouich et al., 1997
BEC4	*Blumeria graminis* f.sp. *hordei*	ARF-GAP proteins	interferes with host vesicle trafficking	Schmidt et al., 2013
AVR2	*Cladosporium fulvum*	RCR3	inhibits cysteine protease	Rooney et al., 2005
AVR4	*Cladosporium fulvum*	Chitin	protects hyphae	van den Burg et al., 2006
Ecp6	*Cladosporium fulvum*	Chitin	inhibits chitin-triggered immunity	de Jonge et al., 2010
CfTom1	*Cladosporium fulvum*	α-Tomatine	detoxification of secondary metabolites	Ökmen et al., 2013
Victorin	*Cochliobolus victoria*	TRX-h5	induces LOV1-mediated cell death	Lorang et al., 2012
ATR1	*Hyaloperonospora arabidopsidis*	unknown	suppresses PTI	Sohn et al., 2007
ATR13	*Hyaloperonospora arabidopsidis*	unknown	suppresses PTI	Sohn et al., 2007
HaRXL44	*Hyaloperonospora arabidopsidis*	MED19a	interferes with SA-triggered immunity	Caillaud et al., 2013
HaRXL96	*Hyaloperonospora arabidopsidis*	unknown	suppresses cell death and callose depositon	Anderson et al., 2012
AVRPiz-t	*Magnaporthe oryzae*	APIP6	suppresses MAMP-triggered immunity	Park et al., 2012
Slp1	*Magnaporthe oryzae*	Chitin	inhibits chitin-triggered immunity	Mentlak et al., 2012
MfAVR4	*Mycosphaerella fijiensis*	Chitin	protects hyphae	Stergiopoulos et al., 2010
Mg1LysM	*Mycosphaerella graminicola*	Chitin	protects hyphae	Marshall et al., 2011
Mg3LysM	*Mycosphaerella graminicola*	Chitin	protects hyphae; inhibits chitin-triggered immunity	Marshall et al., 2011
AVR1	*Phytophthora infestans*	Sec5	stabilizes Sec5 to modulate exocytosis	this thesis
AVR3a	*Phytophthora infestans*	CMPG1	stabilizes CMPG1 to prevent host cell death	Bos et al., 2010
AVRblb1	*Phytophthora infestans*	LecRK-I.9	interferes with PM-CW adhesion	Bouwmeester et al., 2011a
AVRblb2	*Phytophthora infestans*	C14	prevents secretion of plant protease C14	Bozkurt et al., 2011
EPI1	*Phytophthora infestans*	P69B	inhibits serine proteases	Tian et al., 2004
EPI10	*Phytophthora infestans*	P69B	inhibits serine proteases	Tian et al., 2005
EPIC1	*Phytophthora infestans*	unknown	inhibits cysteine proteases	Tian et al., 2007
EPIC2B	*Phytophthora infestans*	PIP1	inhibits cysteine proteases	Song et al., 2009
IPI-O4	*Phytophthora infestans*	Rpiblb1	supresses ETI triggered by IPI-O1/Rpiblb1	Chen et al., 2012
PexRD2	*Phytophthora infestans*	MAPKKKε	suppresses plant immune signaling	King et al., 2014
Pi03192	*Phytophthora infestans*	NTP1/NTP2	prevents nuclear localization of NAC TFs	McLellan et al., 2013
PSE1	*Phytophthora parasitica*	unknown	modulates host auxin physiology	Evangelisti et al., 2013
AVR1b-1	*Phytophthora sojae*	unknown	suppresses BAX triggered cell death	Dou et al., 2008
AVR3b	*Phytophthora sojae*	unknown	acts as an ADP-ribose/NADH pyrophosphorylase	Dong et al., 2011
Avh331	*Phytophthora sojae*	unknown	modulates host MAPK signaling pathways	Cheng et al., 2012
GIP1	*Phytophthora sojae*	β-1,3-Glucanases	inhibits glucanases	Rose et al., 2002
PsAvh163	*Phytophthora sojae*	unknown	suppresses callose depositon	Anderson et al., 2012
PSR1/PSR2	*Phytophthora sojae*	unknown	suppresses host plant RNA silencing	Qiao et al., 2013
RTP1p	*Uromyces fabae/U. striatus*	Host proteases	inhibits proteases	Pretsch et al., 2013
Cmu1	*Ustilago maydis*	Cm2	changes host metabolic status	Djamei et al., 2011
Pep1	*Ustilago maydis*	POX12	inhibits host peroxidase activity	Hemetsberger et al., 2012
Pit2	*Ustilago maydis*	CP2, CP1A/B, XCO2	inhibits cysteine proteases	Mueller et al., 2013
Tin2	*Ustilago maydis*	ZmTTK1	promotes anthocyanin biosynthesis	Tanaka et al., 2014

Vesicle trafficking is one of the conserved hubs targeted by pathogen effectors

Upon pathogen infection, the host plant activates vesicle trafficking to secrete antimicrobial compounds, including cell wall components, proteins and secondary metabolites to either strengthen the cell wall, to form physical barriers or to directly inhibit pathogen growth. As shown by Wang et al. (2005), silencing of genes that are involved in protein secretion could result to a reduction in PR-1 secretion and thus lead to reduced resistance to the bacterial pathogen *Pseudomonas syringae*. Similarly, Ostertag et al., (2013) showed that silencing of a putative *exo70F* gene and several Golgi-membrane trafficking related genes reduces the penetration resistance to the barley powdery mildew *Blumeria graminis*. In order to colonize the host plant, plant pathogens have evolved ways to evade this basal defense by manipulating host polarized secretion via its secreted effectors. There are few studies showing that pathogen effectors are able to target proteins involved in host vesicle trafficking pathways. For example, the bacterial pathogen *P. syringae* secretes the type III effector HopM1 to target and degrade the *AtMIN7* encoded host protein ARF-GEF, a regulator involved in vesicle formation, and is thus able to suppress polarized plant defense and promote pathogen colonization (Nomura et al., 2006). Similarly, the *Blumeria graminis* effector BEC4 was found to target ARF-GAP, which possibly interferes with host vesicle trafficking (Schmidt et al., 2013). *Alternaria carthami* secretes the nonproteinaceous compound Brefeldin A (BFA) to inhibit vesicle trafficking (Driouich et al., 1997). Plant pathogens can also inhibit host focal secretion by employing effector proteins that bind to immune-related proteins to stop their secretion. For instance, *P. infestans* RXLR effector AVRblb2 is able to target the defense protease C14 to suppress its focal secretion (Bozkurt et al., 2011). These findings indicate that vesicle trafficking is one of the conserved hubs targetted by pathogen effectors to suppress host immunity.

In the *P. infestans*-potato pathosystem, we have identified the exocyst subunit Sec5 as a host target of *P. infestans* RXLR effector AVR1 (**Chapter 4**). As a virulence factor, AVR1 specifically interacts with Sec5 and suppresses basal defense in the host, such as callose deposition. Sec5 plays an important role in secretion of PR-1 and callose deposition, and is thus essential for plant defense against *P. infestans*. AVR1 is likely able to hamper this immune secretion by targeting the host exocyst component Sec5. Our findings have unravelled a new strategy that *P. infestans* uses to hamper the host secretion system, and that is

by targeting and stabilizing the exocyst component protein Sec5 thus interfering with host exocytosis.

Sec5 seems specifically involved in R1-mediated defense responses since R1/AVR1 triggered HR was altered in Sec5 silenced plants but not R3a/AVR3a and Rx/CP triggered HR (**Chapter 4**). However, we think that Sec5 is involved in the downstream signalling of R1-mediated resistance instead of being a guardee of R1. First of all, the development of R1/AVR1 triggered HR on *Sec5*-silenced plants is around 60% compared to 100% on control plants, which is a rather mild reduction compared to other guardees as for example, BSL1, a protein associated with R2 and AVR2. More than half of the HR triggered by R2/AVR2 is reduced on *BSL1*-silenced plants (from 80% in the control to 30 to 40% on the *BSL1*-silenced plants) (Saunders et al., 2012). Secondly, Sec5 is also involved in cell death responses triggered by other effectors e.g. Crinklers (CRNs) (**Chapter 5**). CRNs are a diverse group of intracellular effectors, several of which were found to localize in the host nucleus and able to promote pathogen colonization (Stam et al., 2013; van Damme et al., 2012; Schornack et al., 2010). CRN2 induces cell death but the downstream signalling of CNR2-triggered cell death is unknown. Preliminary data show that *Sec5*-silenced plants also display reduced HR triggered by Cf4/AVR4 and Cf9/AVR9 (data not shown). This might be due to the fact that secretion of Cf receptor proteins to the plasma membrane is reduced in *Sec5*-silenced plants. Altogether, our results point to a general role for Sec5 in downstream signalling of plant immunity including R1-mediated immunity.

In contrast to AVR1, AVR1-like is not able to interact with Sec5. Both AVR1 and AVR-like promote *P. infestans* colonization, but only AVR1 is able to suppress callose deposition and CRN2-induced cell death which is dependent on Sec5. AVR1-like is not able to do so **(Chapters 4** and **5)**. Domain deletion assays showed that the C-terminal T-region of AVR1 is essential for promoting *P. infestans* colonization and is also needed to suppress callose deposition and CRN2-triggered cell death, processes depending on Sec5 (**Chapter 5**). We therefore analysed whether the T-region of AVR1 is also essential to interact with Sec5. Indeed deletion of the T-region abolished the interaction between AVR1 and Sec5, but in addition deletion of the Y motif also hampered the interaction, indicating that both the Y motif and T-region are essential for Sec5 interaction.

The nucleocytoplasmic partitioning of AVR1 (**Chapter 3**) raised the question which subcellular localization of AVR1 is required for suppression of CRN2-induced cell death. We could show that cytoplasmic localized AVR1 ($AVR1^{NES}$)

is able to suppress CRN2-induced cell death, while nuclear localized AVR1 ($AVR1^{NLS}$) loses this ability (**Chapter 3**). Since CRN2-triggered cell death is dependent on Sec5, and Sec5 is a cytoplasmic protein, it is likely that the loss of cell death suppression by $AVR1^{NLS}$ is due to the fact that it can no longer physically interact with Sec5. The finding that $AVR1^{nls}$ does not behave as expected (i.e. similar to unmodified AVR1), could be due to the fact that NLS function is not completely abolished in the mutated NLS version so that the AVR1 is still primarily localized in the nucleus. This could be checked by measuring the ratio of the fluorescence intensity in the cytoplasm and the nucleus.

The role of exocyst subunits in plant defense against pathogens

The exocyst is a protein complex consisting of eight subunits. It was first identified in yeast, and is known to function mainly in exocytosis. However its role in plants is hardly studied. So far, several studies have reported that the exocyst plays a role in plant development and polarized secretion including polar auxin transport (Drdova et al., 2013; Hala et al., 2008; Fendrych et al., 2010). In addition, a possible role in plant-microbe interaction is also emerging. A few recent studies have shown that some exocyst subunits are required for plant defense against bacterial pathogens or plant resistance to fungal penetration, i.e. Exo70B1, Exo70B2, Exo70H1 and Exo70F-like (Pecenkova et al., 2011; Stegmann et al., 2012; Stegmann et al., 2013; Ostertag et al., 2013).

Besides Sec5 we analysed the role of other exocyst subunits in defense against *Phytophthora* pathogens such as *P. infestans* and *P. capsici* (**Chapter 6**). We observed that most of the exocyst subunits are required for defense against *P. infestans* in *N. benthamiana*, with the exception of a few EXO70 paralogs, such as EXO70C and EXO70D. In addition, our results showed that most of the exocyst subunits are required for callose deposition. This is likely due to the fact that the enzymes for callose synthesis could be transported via exocytosis. Silencing of exocyst subunits would hamper the localization of callose synthases to the plant plasma membrane and would result in impaired callose deposition. Since the exocyst is likely involved in exocytosis which is a basal cellular process, it is not surprising that silencing of the genes encoding the exocyst subunits altered plant development. However, altered plant development does not necessarily affect defense against pathogens. We found that silencing of *Exo70D* in *N. benthamiana* results in smaller plants similar to silencing of *Sec3* and *Sec6* (**Chapter 6**). However, the *Exo70D*-silenced plants were more resistant to *P. infestans* instead

of more susceptible as was the case with *Sec3* and *Sec6*. As a result the smaller plants were not always susceptible to *P. infestans*, and there was no clear link between altered plant development and plant susceptibility. We also determined that there is functional redundancy in plant development between different subunits, for example, Sec15A and Sec15B, the Exo84s and some of the Exo70s. Exo70 is the only largely expanded subunit in plants. Half of the Exo70 subfamily genes do not play a role in plant development, and few of them are also not involved in plant defense against *Phytophthora* pathogens. This indicates that the Exo70 paralogs in plants are functionally diversified. Some of the *Exo70* subunit genes could play a role in plant development, while others may be involved in other host processes, such as host immunity or polarized secretion.

Functional redundancy of pathogen effectors

Our results together with previous reports, suggest that different pathogens secrete variable effectors to target conserved hubs in host immunity. These conserved hubs include host proteases, chitin (for pathogenic fungi), secondary metabolites, host hormone signalling, the host transcriptional machinery, host MAPK pathways, and host vesicle trafficking pathways (Table 1). When zooming in on a specific pathogen, it is not surprising that some of its effectors are functionally redundant. A study on the bacterial type III effectors of *P. syringae* pv. *phaseolicola* 1448a has identified 27 effectors in the secretome of this pathogen that are translocated to the host cell (Zumaquero et al., 2010). It was shown that 4 out of the 27 effectors (i.e. AvrB4-1, AvrB4-2, HopW1-1 and HopW1-2) that function as virulence factors, are functionally redundant as demonstrated by analysing single and double mutant effector strains (Zumaquero et al., 2010). For a specific plant pathogen, redundancy of effectors would imply that several effectors target different steps in the same host immune signalling pathway or even target proteins in the same essential hub in plant immunity. A well-known host protein that is perturbed by different effectors from the same pathogen is Arabidopsis RIN4 (Grant et al., 2006). RIN4, a negative regulator of plant basal defense responses, is targeted by three different *P. syringae* type III effectors, i.e. AvrRpt2, AvrRpm1, and AvrB (Kim et al., 2005). The fungal pathogen effectors AVR4 and ECP6 from *C. fulvum* and Mg1LysM and Mg3LysM from *Mycosphaerella graminicola* all bind with fungal chitin either to protect fungal hyphae against chitinase or inhibit chitin-triggered immunity (van den Burg et al., 2006; de Jonge et al., 2010; Marshall et al., 2011). There are multiple examples of effectors from

the same pathogen that target different steps in plant immune signalling. For example, the effectors HopAI1 and HopF2 from *P. syringae* are found to interfere with different steps downstream in the plant PTI signalling pathway (Zhang et al., 2007; Wang et al., 2010; Zhou et al., 2014). Another recent study has shown that several *P. infestans* RXLR effectors are able to suppress early flg22-induced immune responses in tomato protoplasts (Zheng et al., 2014). Previously the exocyst component protein Sec3 was picked up as a putative host target of the *P. infestans* RXLR effector AVR3a in yeast two hybrid screening (Bos et al., 2010). In addition, Bozkurt et al. (2011) have shown that *P. infestans* AVRblb2 is able to hamper the secretion of the host cysteine-rich protein C14. As described in this thesis we have solid evidence for Sec5 being a host target of the RXLR effector AVR1. All together these findings support the hypothesis that some of the RXLR effectors from *P. infestans* are functionally redundant and likely target different steps in the host vesicle trafficking pathway to hamper host secretion of antimicrobial compounds to infection sites. Further investigation of RXLR effector biology will likely identify other host targets that function at different levels of vesicle trafficking. This will help us to increase our insight into mechanisms underlying effector-modulated host susceptibility and the role of vesicle trafficking in plant defense.

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Summary

Late blight, caused by the oomycete *Phytophthora infestans,* is one of the most devastating potato diseases worldwide. To successfully colonize its host, *P. infestans* secretes a plethora of RXLR effectors that translocate into host cells to modulate plant defense. The RXLR effectors form the largest and most diverse effector family in oomycete plant pathogens, and include several that were demonstrated to trigger host resistance mediated by intracellular host immune receptors. **Chapter 1** is a summary focussing on the molecular mechanisms underlying host–pathogen interactions. It introduces the multi-layered innate immune system of plants, as well as the strategies that pathogens exploit to circumvent and suppress host defense. Furthermore, it highlights the importance of vesicle-trafficking during plant defense.

The central subject of **this thesis** is AVR1, one of the race-specific avirulence (AVR) factors of *P. infestans*. AVR1 triggers plant resistance mediated by its corresponding potato Nucleotide-binding Leucine-rich repeat (NLR) resistance protein R1. *P. infestans* isolates that are avirulent on *R1*-containing potato cultivars always contain *AVR1*, while virulent isolates lack *AVR1* but contain a related gene that we baptized as *AVR1-like.* AVR1 has all hallmarks of a typical RXLR effector; it contains a signal peptide, an RXLR domain and a C-terminal effector domain that contains two W motifs and one Y motif. In addition, it has, at the very end a stretch of 38 amino acids in length that we named the Tail (T)-region. AVR1-like, or in short A-L, shares high sequence similarity with AVR1. However, due to a premature stop codon the 38 amino acid T-region is missing.

Chapter 2 explores the conserved motifs and regions in the C-terminal effector domain of AVR1 that are required to trigger R1-mediated hypersensitive response (HR). Various truncated and chimeric constructs of AVR1 and A-L were generated and assayed for their ability to elicit R1-mediated HR. Results show that the T-region of AVR1 plays an important role in HR activation. Furthermore, we revealed that R1 recognizes two epitopes in AVR1, one located in the C-terminal region containing the conserved W and Y motifs, and one comprised by the T region.

In **Chapter 3** the subcellular localization of AVR1 and R1 was investigated. Both were demonstrated to be nucleocytoplasmic proteins. We artificially modified the nucleocytoplasmic partitioning of AVR1 and R1 using nuclear localization and export signals (NLS/NES), and studied the effect on R1-AVR1 recognition. This

revealed that nuclear localization of both AVR1 and R1 is important to induce R1-mediated immunity. In addition, we showed that AVR1-mediated suppression of CRN2-induced cell death is dependent on cytosolic localization of AVR1.

In **Chapter 4**, we investigated how AVR1 modulates host defense. In a yeast two-hybrid screening we identified the exocyst subunit Sec5 as a host target for AVR1. Interaction between AVR1 and Sec5 was confirmed *in planta* by co-immunoprecipitation and bimolecular fluorescent complementation. Although A-L shares high sequence similarity with AVR1, we found that it is not able to interact with Sec5. Sec5 was shown to be required for proper plant defense against *P. infestans*. The role of Sec5 in plant response upon pathogen attack was further supported by its role in callose deposition and in secretion of the pathogenesis-related protein PR-1, which indicates that Sec5 plays a crucial role in vesicle trafficking during host defense. AVR1 is able to suppress callose deposition while A-L is not, which suggests that *P. infestans* manipulates host vesicle trafficking by secretion of AVR1 to target Sec5. Overall, our findings unravelled a novel strategy that oomycete pathogens exploit in order to modulate host defense.

In **Chapter 5** we further analysed the potential virulence activities of AVR1 and A-L. Both AVR1 and A-L were able to promote *P. infestans* colonization, indicating that both are genuine *P. infestans* virulence factors. Moreover, AVR1 was found to suppress not only callose deposition, but also Sec5-dependent cell death induced by the *P. infestans* elicitors INF1 and CRN2. In contrast, A-L was neither able to suppress Sec5-dependent nor Sec5-independent cell death. The conserved C-terminal motifs and regions required for virulence activity of AVR1 were investigated using AVR1 truncated constructs. In addition, the conserved C-terminal motifs and regions of AVR1 required for Sec5 interaction were studied by Y2H assays. Although the T-region of AVR1 was found to be sufficient to facilitate *P. infestans* colonization and suppression of CRN2-induced cell death, it could not fully accommodate the interaction of AVR1 with Sec5. Instead, both the Y motif and the T-region of AVR1 appear to be required for Sec5 targeting.

Next to Sec5, the role of other exocyst subunits in *Phytophthora* resistance was studied (**Chapter 6**). The evolutionary relationships of exocyst subunits from three Solanaceous plants, i.e. *Nicotiana benthamiana*, tomato and potato, were investigated in comparison to their Arabidopsis orthologs. Virus-induced gene silencing in *N. benthamiana* of the majority of the exocyst subunit genes (*exo84*s

were not yet included) showed that, except for some Exo70 members, all other tested exocyst subunits are required for plant defense against *P. infestans* and callose deposition. In addition, all of the analysed exocyst subunit gene-silenced tomato plants showed gain of susceptibility to both *P. infestans* and *Phytophthora capsici*.

In **Chapter 7**, our findings obtained in this thesis on the mechanisms of AVR1-triggered host immunity and susceptibility are discussed in a broader perspective with emphasis on the current developments in the field of effector biology.

Samenvatting

De aardappelziekte, die veroorzaakt wordt door de oömyceet *Phytophthora infestans*, is een van de meest verwoestende ziektes in de aardappelteelt wereldwijd. Om zijn gastheer met succes te koloniseren produceert *P. infestans* een breed spectrum aan RXLR effectoren waarmee de ziekteverwekker afweerreacties in de plant kan onderdrukken. De RXLR effectoren vormen de grootste en meest diverse effectorfamilie in oömyceten. Voor verscheidende RXLR effectoren is aangetoond dat ze gastheerresistentie initiëren door activatie van intracellulaire immuun-receptoren in de plant. In **Hoofdstuk 1** wordt beschreven hoe planten zich verdedigen tegen ziekteverwekkers en welke strategieën ziekteverwekkers exploiteren om afweer te omzeilen. Daarnaast wordt het belang van transport via vesikels tijdens de verdediging van planten belicht.

Het centrale onderwerp in **dit proefschrift** is de RXLR effector AVR1, een van de cultivar-specifieke avirulentie (AVR) factoren van *P. infestans*. AVR1 activeert resistentie in aardappelplanten die het resistentie-gen *R1* bevatten dat codeert voor een 'nucleotide binding leucine-rich repeat-type' (NLR) eiwit. *P. infestans* isolaten die niet in staat zijn R1-bevattende aardappelrassen te infecteren, en dus avirulent zijn op R1 planten, hebben allen het *AVR1* gen. Isolaten daarentegen die virulent zijn op R1 planten missen *AVR1* maar hebben een verwant gen dat we *AVR1-like* hebben genoemd. AVR1 heeft alle kenmerken van een RXLR effector; het bevat een signaalpeptide, een RXLR domein en een C-terminaal effector domein met twee W-motieven en een Y-motief. Daarnaast heeft AVR1 een C-terminale staart van 38 aminozuren die aangeduid wordt als de T–regio. AVR1-like heeft op DNA- en eiwitniveau veel overeenkomsten met AVR1, maar mist de T-regio.

In **hoofdstuk 2** onderzochten we welke geconserveerde motieven en regio's in het C-terminale deel van AVR1 essentieel zijn voor de activatie van R1-geïnduceerde celdood. Verscheidene verkorte en chimere versies van AVR1 en AVR1-like werden gegenereerd, en getest voor het vermogen om R1-afhankelijke celdood te induceren. Hieruit bleek dat de T-regio van AVR1 weliswaar een belangrijke rol speelt in de activatie van celdood maar dat R1 naast de T-regio een tweede epitoop in AVR1 herkent die zich in het C-terminale deel bevindt met de geconserveerde W- en Y-motieven.

In **hoofdstuk 3** werd de subcellulaire lokalisatie van AVR1 en R1 geanalyseerd. Beide eiwitten werden zowel in de celkern (nucleus) als in het cytoplasma van de plantencel aangetroffen. Door gebruik te maken van zogenaamde nucleus-lokalisatie en -export signalen (NLS/NES), waren we in staat om de verdeling van AVR1 en R1 over de celkern en het cytoplasma te beïnvloeden om zodoende het effect op de R1-AVR1 herkenning te bestuderen. Hieruit bleek dat R1-afhankelijke immuniteit alleen geïnduceerd wordt als AVR1 en R1 beide in de celkern van de gastheercel aanwezig zijn. Bovendien toonden we aan dat AVR1-afhankelijke onderdrukking van CRN2-geïnduceerde celdood alleen optreedt als AVR1 in het cytoplasma aanwezig is.

In **hoofdstuk 4** onderzochten we hoe AVR1 plantenafweer moduleert. In een 'yeast two hybrid (Y2H) screening' werd de exocyst-subeenheid Sec5 geïdentificeerd als doelwit van AVR1 in de plantencel. *In planta* interactie tussen AVR1 en Sec5 werd experimenteel bevestigd door co-immunoprecipitatie en bimoleculaire fluorescentie complementatie. Ondanks de hoge sequentiegelijkenis tussen AVR1-like en AVR1, bleek AVR1-like niet met Sec5 te interacteren. Dat Sec5 een rol speelt in plantenafweer bleek uit de waarneming dat Sec5 essentieel is voor een optimale resistentie tegen *P. infestans*, en bovendien bijdraagt aan de afzetting van callose en de secretie van het pathogeniciteit-gerelateerde planteneiwit PR-1. Dit laatste wijst erop dat Sec5 een cruciale rol speelt in transport van afweercomponenten via vesikels. AVR1 kan callose-afzetting onderdrukken, terwijl AVR1-like dit niet kan, en dit suggereert dat *P. infestans,* door middel van het uitscheiden van AVR1, transport via vesikels manipuleert. Deze bevindingen onthullen een nieuwe strategie die plantenpathogene oömyceten inzetten om afweer te moduleren.

In **hoofdstuk 5** onderzochten we de potentiële activiteiten van AVR1 en AVR1-like in virulentie. Expressie in *Nicotiana benthamiana* van zowel AVR1 als AVR1-like bleek infectie te bevorderen en dit geeft aan dat beide functioneren als volwaardige *P. infestans* virulentiefactoren. Bovendien bleek AVR1 niet alleen callose-afzetting te onderdrukken, maar ook Sec5-afhankelijke celdood geïnduceerd door de *P. infestans* elicitors INF1 en CRN2. AVR1-like bleek echter niet in staat celdood te onderdrukken. Verscheidene verkorte en chimere versies van AVR1 en AVR1-like werden getest voor het vermogen om virulentie te bevorderen, celdood te onderdrukken en te interacteren met Sec5 in Y2H assays. Hoewel de T-regio van AVR1 voldoende bleek te zijn om *P. infestans* kolonisatie en onderdrukking van

CRN2-geïnduceerde celdood te bevorderen, was de T-regio alleen niet voldoende om interactie met Sec5 te bewerkstelligen. Hiervoor bleek zowel de T-regio als het Y-motief nodig te zijn.

Naast Sec5, werd ook de rol van andere exocyst-subeenheden in *Phytophthora* resistentie bestudeerd (**hoofdstuk 6**). De evolutionaire verwantschap tussen exocyst-subeenheden van Arabidopsis en de drie nachtschadeachtigen *N. benthamiana*, tomaat en aardappel werd geanalyseerd. Met behulp van 'virus-induced gene silencing' in *N. benthamiana* werd de functie van de exocyst subunit genen (met uitzondering van exocyst subunit Exo84) onderzocht. Hieruit bleek dat verschillende van de geteste exocyst-subeenheden vereist zijn voor een optimale afweer tegen *P. infestans* en voor callose-afzetting. Ook in tomaat bleek 'virus-induced gene silencing' van verscheidende exocyst-subeenheden te resulteren in verhoogde gevoeligheid voor zowel *P. infestans* als *Phytophthora capsici*.

In **hoofdstuk 7**, worden de in dit proefschrift verkregen bevindingen over de mechanismen van AVR1-geïniteerde immuniteit en gevoeligheid besproken in een breder perspectief met nadruk op de actuele ontwikkelingen op het gebied van effectorbiologie.

Acknowledgements

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The second person I want to thank is Klaas, my daily supervisor and co-promoter. Klaas, you are a very interesting person who knows everything; not only about science, but also about history, and most importantly birds. You were very helpful and always made time to help me. Thank you very much for the discussions we have had about my experiments, and for your suggestions and comments on my manuscripts. I am always amazed by your magic ability in finding all those small mistakes in my manuscripts, even an extra space or a different letter font. You persuaded me that you have the sharpest eyes in the world and I am wondering if that is the cause or the consequence of being a birdwatcher.

The Pinf group is a nice and close group, and all its members are very pleasant to work with. Rob, you are a very kind person. You helped me a lot, especially during the first few months when I was here. You were so kind to share with me many tips and tricks on basic molecular techniques. Thanks as well for helping me out with the ATTAs. I still remember that we once worked together in the greenhouse until the lights went off.

Harold, you are very knowledgeable and willing to share your experiences. Thanks for those minor workshops your have held. Thanks for being so friendly in life and picky in science. Chenlei, thanks for your help in the lab. I had many questions, and therefore thanks for being so patient. From Lisha, Chunxu and Xu, I have learned that a good scientist can also be a good cook too. When celebrating the Chinese spring festival at Lisha's or Chunxu's place your authentic Chinese food rescued me from homesickness. I want to express my great appreciation to my dear friend Yan. Thank you for helping me to adjust to the life in Wageningen and thank you for your company in the past four years. We are two Chinese girls with distinct personalities but still we resemble each other in many things. I am not going to list them here, but you know we share happiness and unhappiness; we support each other, and we will even defend our thesis on the same day. I wish you all the best with both, your career and your personal life. Xu and Wei, thanks for your suggestions, encouragements and jokes, which helped me to grow up. Chara, Johan, Elysa, Natalie, Huayi, thanks for the nice time at our pinf dinners and pinf trips. Johan is a good driver; I got to know this during our PhD trip to Germany. Elysa, thanks for organizing our pinf trips. We went to the seaside, which was my first time to see the sea. This experience changed my previous impression of the sea which was based on television or from looking outside from an airplane. Elysa, I am

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In Yangling, China, I first want to thank my supervisor Prof. Dr Gong, thank you very much for your supervision. Prof. Gong, you guided me towards science, there is no word here which can express my thanks to you. Thanks for your understanding and support during the past seven years. Dr Rugang, Minghui, Dawei, Wei Huang and Zheng Li, thank you for your encouragement and helpful discussions we have had. I am very happy to have so many lab-siblings in the group of Prof. Gong. Our respective sister Jun'e and brother Yanxu and other siblings Yingli, Jiaojiao, Huaijuan, Weili, Cui, Jinghao, Meng….. thanks for helping me with document works and I really enjoyed the time we have had together.

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Finally, I express my sincere acknowledgements to my funding organization China Scholarship Council (CSC) for offering me a personal fellowship to pursue my PhD degree at Wageningen University.

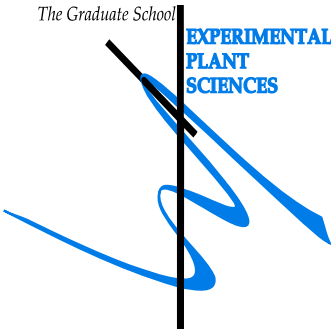
Yu Du, 23rd October, 2014, Wageningen

Curriculum Vitae



Yu Du was born on July 20th, 1984, in Chang'an district of Xi'an city, China. In 2003, after her graduation from the high school in Chang'an, she started her four-year bachelor study in horticulture at Northwest A&F University in Yangling, China. Four years later, in 2007, she obtained her bachelor's degree and in the same year she joined the accelerated Master-Doctor Program in plant breeding and biotechnology at Northwest A&F University. For her MSc thesis research she studied *Phytophthora capsici*-pepper interactions. In November 2008, Yu Du was awarded with the honour 'excellent postgraduate student' for her performance in the compulsory courses in the first year. In 2010, she obtained a personal fellowship from the China Scholarship Council (CSC) which enabled her to extend her study at the Laboratory of Phytopathology at Wageningen University, The Netherlands, under the supervision of Dr Klaas Bouwmeester and Prof. Dr Francine Govers. In September 2010 she started her research training in Wageningen where she focussed on identifying host targets of the *Phytophthora infestans* RXLR effector AVR1. Early 2013 she joined the PhD programme at Wageningen University and enrolled in the Graduate School Experimental Plant Sciences. During her PhD study, she participated in four international congresses and was invited to give an oral presentation at three of them. The results of her PhD research are described in this thesis. The five experimental chapters are in preparation for publication.

Education Statement of the Graduate School Experimental Plant Sciences



Issued to: **Yu Du**
Date: **24 November 2011**
Group: **Phytopathology**
University: **Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Sec5a putative host target of the Phytophthora infestans RXLR effector Avr1	Jun 17, 2011
► **Writing or rewriting a project proposal**	
Activity and host targets of Phytophthora RXLR-effectors	2011
► **Writing a review or book chapter**	
► **MSc courses**	
Plant-Microbe Interactions (PHP 30306)	2011
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*10.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Wageningen UR	May 20, 2011
EPS PhD student day, University of Amsterdam	Nov 30, 2012
EPS PhD student day, Leiden University	Nov 29, 2013
► **EPS theme symposia**	
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents, and Willie Commelin Scholten Day, University of Amsterdam	Feb 03, 2011
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents, and Willie Commelin Scholten Day, Wageningen University	Feb 10, 2012
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents, and Willie Commelin Scholten Day, Utrecht University	Jan 24, 2013
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents, and Willie Commelin Scholten Day, University of Amsterdam	Feb 25, 2014
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04 & 05, 2011
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02 & 03, 2012
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 22 & 23, 2013
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 14 & 15, 2014
► **Seminars (series), workshops and symposia**	
Invited seminar David Baulcombe	Sep 27, 2010
Invited seminar Kirsten Bomblies	Nov 18, 2010
CBSG Technology Symposium "Adcances in life-science Technologies"	Nov 25, 2010
Invited seminar Rosie Bradshaw	Aug 04, 2011
Symposium 'New Developments in Plant Endomembrane Biology" VU University, Amsterdam	Jun 15, 2012
Invited seminar David G. Robinson	Sep 27, 2012
Rob Goldbach Memorial Lecture by David Baulcombe	Oct 10, 2012
Mini symposium ‘Frontiers in Plant Morphogenesis’	Nov 13, 2012

Invited seminar Larry Griffing	Nov 16, 2012
CBSG Workshop Genome Mining	Dec 13, 2012
CBSG Proteomics Hotel meeting	Jan 18, 2013
Mini-symposium 'Intraspecific Pathogen Variation - Implications and Opportunities'	Jan 22, 2013
Invited seminar Andrew sugden	Feb 08, 2013
EPS Flying Seminar Detlef Weigel	Feb 27, 2013
Gabino Sanchez Perez, Plant Sciences Seminar	Mar 12, 2013
Invited seminar Marc Boutry	Mar 13, 2013
Mini symposium 'Integrative and Comparative Genomics of Pathogenic Microorganisms'	May 06, 2013
EPS Flying Seminar Howard S. Judelson	May 07, 2013
EPS Flying Seminar Rays H.Y. Jiang	May 07, 2013
Invited seminar Brian Staskawicz	May 21, 2013
Invited seminar Hanhui Kuang	Sep 11, 2013
Invited seminar of Eric Schranz	Nov 21, 2013
Invited seminar Olga del Pozo	Feb 20, 2014
Invited seminar Jeroen Mesters	Mar 31, 2014
Invited seminar Sophien Kamoun	May 28, 2014
Invited seminar Yuanchao Wang	Jul 16, 2014
► **Seminar plus**	
► **International symposia and congresses**	
The 3rd International Conference on Biotic Plant Interactions (ICBPI), Yangling, Shaanxi, China	Aug 18-22, 2013
The SUSTAIN COST Action meeting, Birnam, Scotland	Oct 09-11, 2013
The SUSTAIN COST Action meeting, Toulouse, France	Apr 09-11, 2014
Oomycete Molecular Genetics Meeting, Norwich, UK	Jul 02-04, 2014
► **Presentations**	
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, The Netherlands (poster)	Apr 02 & 03, 2012
CBSG Pathogenomics meeting (oral)	Oct 25, 2012
EPS Theme 2 Symposium/Willie Commelin Scholten Day (oral)	Jan 24, 2013
Presentation at Yangling Northwest A and F Univercity (oral)	Mar 19, 2013
The 3rd International Conference on Biotic Plant Interactions (ICBPI) (poster)	Aug 18-22, 2013
The SUSTAIN COST Action meeting (oral)	Oct 09, 2013
EPS PhD student day (oral)	Nov 29, 2013
The SUSTAIN COST Action meeting (oral)	Apr 11, 2014
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, The Netherlands (poster)	Apr 14 & 15, 2014
Oomycete Molecular Genetics Meeting (oral)	Jul 02,2014
► **IAB interview**	
Meeting with a member of the International Advisory Board of the Graduate School EPS	Nov 14, 2012
► **Excursions**	
Subtotal Scientific Exposure	*23.1 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Advanced light microscopy facilities at Wageningen UR	Jun 13, 2013
7th Utrecht PhD Summerschool on Environmental Signaling	Aug 26-28, 2013

►	**Journal club**	
	literature discussion in P. infestans group at Wageningen UR	2010-2014
►	**Individual research training**	
	Subtotal In-Depth Studies	*4.2 credits**

4) Personal development	*date*
► **Skill training courses (highly recommended)**	
Wageningen UR Digital Library Introduction	Oct 07, 2010
Workshop for Phytopathology search the digital library efficiently	Jan 25, 2011
CBSG Matchmaking event	Oct 18, 2012
WGS course 'Mobilising your-Scientific-network(MSN)'	Jun 12 & 19, 2013
WGS course 'Scientific Writing'	Oct 15-18, 2013
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*2.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**40.6**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	
** A credit represents a normative study load of 28 hours of study.*	

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Cover images. At the front from left to right: *Nicotiana benthamiana* wild type plants and plants silenced for Sec5 and SGT1. At the back from left to right: the close proximity of AVR1 and Sec5 shown by BiFC and the subcellular localization of AVR1.

Cover and layout design: Yu Du and Guangzhao Liu

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