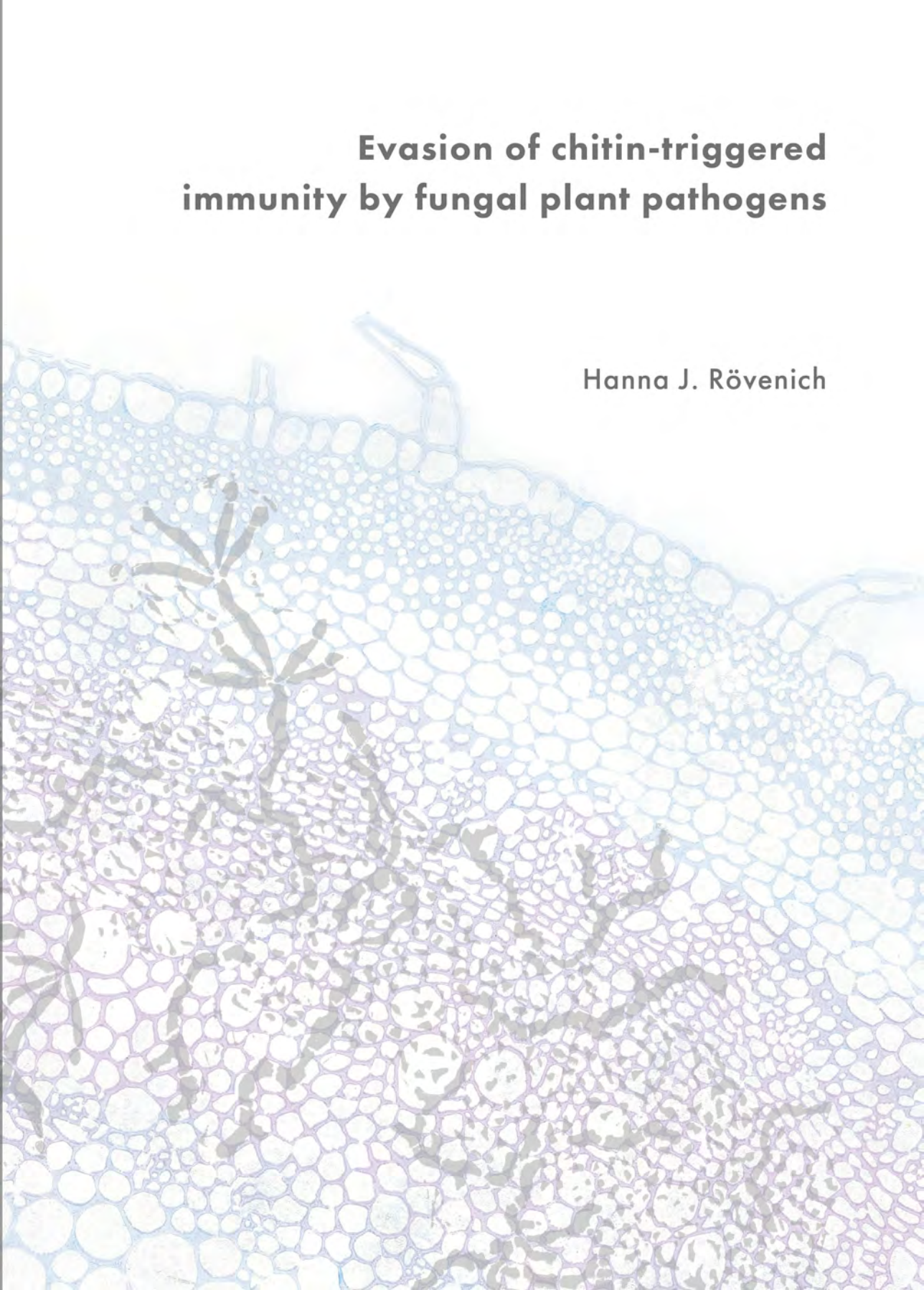
Evasion of chitin-triggered immunity by fungal plant pathogens

Hanna J. Rövenich



Propositions

1. VdAve1 is an effector protein with dual virulence function.
   (this thesis)

2. Evolution of filamentous microbes toward suppression of glycan-triggered immunity is a basic requirement for establishment in any niche.
   (this thesis)

3. The technologies that are being developed by environmental engineers and nanoscale scientists to harvest the energy generated by plants during photosynthesis have the potential to revolutionize the energy market.

4. The next step to enhance our understanding of the mechanisms underlying host-microbe interactions will be to integrate the knowledge on host-associated microbiomes.

5. Courses on science communication and outreach activities should be core requirements of each scientist's education.

6. Learning to speak foreign languages as adults is a matter of mindset.

Propositions belonging to the thesis, entitled

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Thesis

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Contents

Chapter 1

General introduction

Conceptual models of plant immunity

Plants establish very intimate, symbiotic relationships with microbes. However, as in animals, microbial colonization of plant hosts only rarely results in disease. This is mainly due to the presence of a complex immune system, which allows plants to survey their environment for the presence of potential pathogens through the activity of plant immune receptors[1].

The interaction between plant immune receptors and pathogen ligands was originally defined in the "gene-for-gene" hypothesis, which postulated that the products of single-dominant plant resistance (*R*) genes recognize the products of corresponding pathogen avirulence (*Avr*) genes to activate race-specific resistance[2,3]. However, this model failed to explain the function of so-called general elicitors, which defied the rules of race-specificity and were recognized by multiple plant species[4]. It was not until the introduction of the "zigzag" model that these seemingly different observations were combined into a single concept describing plant immunity[5]. In this model, the first line of defence is governed by cell surface-localized pattern recognition receptors (PRRs) that detect pathogen-associated molecules patterns (PAMPs) to activate broad-spectrum disease resistance, called PAMP-triggered immunity (PTI). As PAMPs include molecules that are present both in pathogenic as well as non-pathogenic microbes[6], they have been renamed microbe-associated molecular patterns (MAMPs), whose recognition leads to the establishment of MAMP-triggered immunity (MTI). In turn, successful microbes employ effector molecules to overcome MTI resulting in effector-triggered susceptibility (ETS). In the second layer of defense, effectors are recognized by intracellular receptors (R proteins) that activate effector-triggered immunity (ETI). Microbes may evade recognition through loss or mutation of recognized effectors or actively suppress ETI using novel effectors. These processes describe the continued coevolution between microbes and their hosts, which has been termed the molecular "arms race".

In addition to successfully incorporating the observations made about general elicitors, the "zigzag" model recognizes the role of effector molecules as contributors to microbial virulence irrespective of the host genotype. However, the strict separation of MTI and ETI layers of immunity in the "zigzag" model does not account for the spatio-temporal continuum underlying plant-microbe interactions[7,8]. In addition, is does not resolve the conceptual conflict that MAMPs are defined from the perspective of the host, whereas effectors are defined from the perspective of the invading microbe[8]. These discrepancies have led to the formulation of the Invasion Model, in which host receptors (referred to as invasion pattern receptors, IPRs) recognize microbe-derived or modified-self ligands (invasion patterns, IPs) that reveal invasion[8]. While any molecule can serve as IP that is detected by an IPR, the probability of a particular ligand-receptor complex to evolve within the framework of host immunity increases with the probability that the ligand retains its function, conservation across organisms, importance in establishment of symbiosis, and accessibility[8].

Main objective and research questions

With the availability of high-quality genome and transcriptome data for many microbial species, the number of identified putative effector molecules has rapidly increased in recent years. However, the functions of most of these effectors, as well as the mechanisms governing their recognition by plant receptors, have largely remained unexplored. The main objective of my doctoral research was to tackle this gap specifically in plant-fungal interactions. By using a combination of physiological, biochemical, and proteomic approaches I addressed the following general questions:

1. How do plants perceive microbe-derived molecules?
2. What are the roles of effector proteins during host plant infection and how do they contribute to virulence of different fungal pathogens?

Study systems

The host model plant tomato

Tomato (*Solanum lycopersicum* L.) is considered one of the most important vegetable crops with a worldwide distribution and a net economic value exceeding $55 billion[9]. After several decades of breeding efforts for the introduction of resistance loci into cultivated accessions, and with the increasing availability of genomic resources, tomato has become a particularly important model crop for the study of plant-pathogen interactions. In addition, it represents an important model species for biological research on the genetic improvement of Solanaceous crops, including pepper, potato and eggplant[10].

The broad host-range pathogen *Verticillium dahliae*

The *Verticillium* genus consist of ten currently recognized fungal ascomycete species whose lifestyles range from saprotrophic to symbiotic[11]. Among the symbionts, *Verticillium dahliae* is the most detrimental pathogen as it is able to infect over 200 dicotyledonous plant species including many crops, such as tomato[12]. It has been estimated that wilt disease caused by *Verticillium* spp. results in billions of dollars of annual economic losses[13,14].

V. dahliae resting structures, called microsclerotia, reside within the soil where they germinate in response to plant root exudates[15]. Emerging hyphae penetrate the host tissue at root tips or at sites of lateral root formation[16]. In susceptible plants, the fungus colonizes the vascular system after invasive hyphae have crossed the root endodermis. Conidia formation and transport throughout the vasculature eventually results in systemic colonization[15]. This causes chlorosis, necrosis and wilting of the host plant. The fungus

then enters a saprotrophic stage during which it colonizes necrotic and senescent plant tissues. At these late infection stages, *V. dahliae* produces large amounts of microsclerotia that are released into the soil upon decomposition of the host plant tissue, where they can survive for 10-15 years[17]. Due to the broad host range of *V. dahliae* and the long-term prevalence of its resting structures in the soil, agricultural practices such as crop rotation do not result in crop protection[15]. Disease control is particularly difficult as fungicides are generally ineffective once the pathogen has entered the vascular system, and soil fumigation has largely been banned due to harmful effects on the environment and/or public health. Therefore, the preferred method for disease control is genetic resistance.

Genetic resistance to *V. dahliae* has been identified in several crop species[12,18-21]. In tomato, the *Ve* locus was described to confer resistance against race 1 but not race 2 isolates of *V. dahliae*[22,23], and has been introduced into most cultivated tomato genotypes[15]. Positional cloning showed that the *Ve* locus contains two closely linked genes, *Ve1* and *Ve2*, both of which encode membrane-bound extracytoplasmic leucine-rich repeat receptor proteins (eLRR-RPs)[24]. While both Ve1 and Ve2 conferred resistance to pathogenic *V. albo-atrum* when expressed in susceptible potato plants, only Ve1 could be confirmed to provide resistance against race 1 isolates in tomato and other plant species[21,24,25].

Cladosporium fulvum, the tomato specialist

In contrast to *V. dahliae*, *Cladosporium fulvum* is a non-obligate biotrophic fungus that causes leaf mold on tomato. On susceptible plants, *C. fulvum* conidia germinate on the abaxial side of a leaf and hyphae enter the plant through open stomata to invade leaf intercellular spaces[26,27]. Despite the lack of feeding structure formation, *C. fulvum* growth seems to rely on the physical contact with host cells in close proximity to vascular tissues. This is thought to be due to the sucrose gradient that is established around the phloem[28,29]. The first disease symptoms appear at approximately one week after the start of infection as pale green or yellow spots on the upper leaf surface as well as white to olive-green patches of mold on the abaxial sides of leaves. These turn brown upon sporulation at 10-14 days. In severe cases, sporulation is associated with leaf wilting and may lead to plant death[26].

Like other plant pathogens, *C. fulvum* employs effector proteins to successfully infect susceptible tomato plants. So far, 13 effectors have been identified and the corresponding genes have been cloned[30-39]. While the three *C. fulvum* effectors Avr2[40-42], Avr4[43-45], and Ecp6[30,46,47] have been functionally characterized, the intrinsic function of most of them remains unknown. In resistant tomato accessions, *C. fulvum* effectors are recognized by *Cf* resistance genes that, like *Ve1* and *Ve2*, encode eLRR-RPs[48]. This triggers immune responses, which ultimately result in a hypersensitive response (HR), a form of localized cell death that halts pathogen growth at the infection site[49]. Most of the *Cf* genes that have been introgressed into cultivated tomatoes originate from wild *Solanum* species and landraces[50]. Even though the use of resistant cultivars has been effective in containing the

pathogen, their intensive cultivation has led to the emergence of novel *C. fulvum* strains capable of overcoming cloned *Cf* receptor genes[51-54].

Thesis outline

Plant hosts employ surface-localized receptor molecules to survey their environment for the presence of potentially harmful microbes. These receptors perceive ligands, which are either microbe-derived or result from microbe-mediated plant manipulation, to activate immunity. In order to circumvent recognition or suppress immune responses, microbes secrete effector proteins that deregulate host physiological processes. The emphasis of the work presented here lies within the identification and functional characterization of tomato receptor proteins involved in microbe recognition, and the effector proteins employed by the fungal pathogens *C. fulvum* and *V. dahliae* to facilitate tomato colonization.

Effectors are not unique to pathogens but are employed by any microbe that encounters immune responses during plant host colonization. Moreover, plant-microbe interactions occur in environments that contain additional microbial partners, which can affect the colonizing microbe as well as the host plant. **Chapter 2** reviews the role of effector molecules secreted by pathogenic filamentous microbes in the suppression of plant immune responses, and proposes their involvement in microbial competition or cooperation to shape plant microbiomes.

Chitin is a major structural component of fungal cell walls. During host colonization chitin recognition results in the activation defense responses that threaten the survival of fungal invaders. The tomato leaf mold pathogen *C. fulvum* has evolved several strategies to prevent chitin recognition during colonization of its tomato host. The *C. fulvum* effector protein Ecp6 has previously been shown to sequester chitin fragments released from the fungal cell wall to suppress their recognition. However, Ecp6 has been hypothesized to additionally interfere with the formation of host chitin receptor complexes required for the activation of chitin-triggered immunity. **Chapter 3** addresses this hypothesis using the model plant species *Arabidopsis thaliana*.

While chitin recognition has been intensely studied in *A. thaliana* and rice, little is known about chitin perception in tomato. In **Chapter 4**, we present a proteomics approach that led to the identification of putative tomato chitin receptor candidates. Subsequent oxidative burst and gene expression assays confirmed that silencing of a single candidate is sufficient to reduce chitin responsiveness in tomato.

V. dahliae is a successful pathogen on hundreds of plant species. Through comparative genomics the effector protein Ave1 has recently been identified as a major virulence factor during *V. dahliae* infection of susceptible tomato and other hosts. Phylogenetic analysis

demonstrated that, in contrast to many other effectors, Ave1 has homologs in several plant pathogens as well as numerous plants. Based on this phylogenetic distribution, it has been hypothesized that Ave1 has been acquired by *V. dahliae* through horizontal gene transfer. **Chapter 5** describes the functional characterization of Ave1, revealing that this effector has functionally diverged from its microbial and plant homologs.

In tomato cultivars that carry the *Ve* locus, recognition of Ave1 by the leucine-rich repeat-containing receptor protein Ve1 results in resistance to *V. dahliae*. The *Ve* locus contains a second gene, *Ve2*, to which no function could be ascribed despite its homology to Ve1. Nonetheless, both Ve1 and Ve2 were shown to bind the adaptor receptor SOBIR1 in a ligand-independent manner. In **Chapter 6**, we use a biochemical approach to further investigate the composition of the Ve1 receptor complex upon ligand binding and address the question of the lack of Ve2 function in *V. dahliae* resistance.

Chapter 7 places the most important findings of this thesis into the broader context of glycan-triggered immunity in plants, and discusses the importance of its evasion by filamentous pathogens.

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Chapter 2

Filamentous pathogen effector functions: of pathogens, hosts and microbiomes

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Abstract

Microorganisms play essential roles in almost every environment on earth. For instance, microbes decompose organic material, or establish symbiotic relationships that range from pathogenic to mutualistic. Symbiotic relationships have been particularly well studied for microbial plant pathogens and have emphasized the role of effectors; secreted molecules that support host colonization. Most effectors characterized thus far play roles in deregulation of host immunity. Arguably, however, pathogens not only deal with immune responses during host colonization, but also encounter other microbes including competitors, (myco)parasites and even potential co-operators. Thus, part of the effector catalog may target microbiome co-inhabitants rather than host physiology.

Introduction

During early microbial colonization stages, plant cell surface-localized pattern recognition receptors (PRRs) recognize microbe-associated molecular patterns (MAMPs), such as fungal chitin, to activate immune responses[1,2]. In order to establish themselves, adapted pathogens secrete effector molecules that deregulate immune responses and facilitate host colonization. Simultaneously, hosts evolve effector recognition by novel receptors that reinstall immunity[1,2]. Consequently, effectors are subject to various selective forces that drive their evolution, leading to diversified effector repertoires between pathogen lineages. Functional characterization of effectors and determination of their contribution to the microbial lifestyle provides insight in relevant processes for host colonization.

Plant pathogen effectors deregulate host immunity in various subcellular compartments

Many pathogens initially enter the plant apoplast, which contains enzymes that hamper microbial colonization. For example, chitinases target fungal cell walls to release chitin fragments that activate immune receptors, leading to further chitinase accumulation to induce hyphal lysis. In turn, fungal pathogens secrete chitin-binding effectors to protect their cell walls and interfere with immune receptor activation[3-6]. The LysM domain-containing Ecp6 effector of the leaf mold fungus *Cladosporium fulvum* can outcompete host receptors through chitin binding with unprecedented ultrahigh (pM) affinity by intramolecular LysM domain dimerization[7]. Additionally, LysM effectors likely interfere with receptor dimerization that is required to activate immune signaling[7-9].

Although effectors that directly target chitinases have not yet been identified, some effectors target other apoplastic hydrolytic enzymes, such as proteases. For example, sequence-unrelated effectors of *C. fulvum*, the oomycete *Phytophthora infestans*, and the parasitic nematode *Globodera rostochiensis* inhibit tomato cysteine proteases including Rcr3[10-12]. The closely related oomycetes *P. infestans* and *P. mirabilis* express an orthologous pair of host protease inhibitor effectors that are subject to positive selection, which was implicated in adaptation to unique protease targets in their respective host plants[13]. Besides protease inhibitors, *P. infestans* secretes the Avrblb2 effector that interferes with protease secretion[14]. The smut fungus *Ustilago maydis* inhibits apoplastic proteases via multiple effectors. While Pit2 directly inhibits cysteine proteases[15], Pep1 induces the maize cystatin CC9 that inhibits apoplastic proteases in turn[16]. Pep1 furthermore inhibits the maize peroxidase POX12 to perturb reactive oxygen species balances[17]. Thus, the plant apoplast is a dynamic battlefield for plant pathogens.

In addition to apoplastic effectors, many pathogens deliver effectors that act inside host cells, although mechanisms that govern their uptake remain controversial[18]. The rice blast fungus *Magnaporthe oryzae* was shown to secrete various effectors that enter rice cells, and even move to non-infected neighboring cells, presumably to prepare these for infection[19]. The AvrPiz-t effector targets proteasome activity through interaction with the RING E3 ubiquitin ligase APIP6, leading to their mutual degradation and suppression of PRR-mediated immunity[20]. Effector diffusion from infected cells into neighboring cells was similarly observed for the *U. maydis* chorismate mutase Cmu1 that targets the shikimate pathway to channel chorismate into the phenylpropanoid pathway, thus adversely affecting salicylic acid (SA) biosynthesis[21]. *U. maydis* furthermore secretes the Tin2 effector to stabilize the maize ZmTTK1 kinase that controls anthocyanin biosynthesis, possibly to suppress tissue lignification[22]. Also the oomycete *Hyaloperonospora arabidopsidis* targets SA signaling by secreting a nuclear-localized effector that interacts with the mediator complex that controls interactions between transcriptional regulators and RNA polymerase[23]. Host transcription is furthermore perturbed by effectors that inhibit transcription factor translocation to the nucleus[24]. Additionally, nuclear-localized effectors may affect host immunity post-transcriptionally by suppressing the biogenesis of small RNAs in the host[25]. Interestingly, *Botrytis cinerea* was recently suggested to deliver even small RNAs into host cells to affect immune responses[26].

Finally, several effectors target host cell death mechanisms, such as *P. infestans* Avr3a and PexRD2. While Avr3a suppresses INF1-triggered cell death by stabilizing the U-box E3 ligase CMPG1 during biotrophic growth, PexRD2 targets the kinase domain of the cell death regulator MAPKKKε[27,28]. During later stages of infection, however, *P. infestans* relies on induction of host cell death as it switches to a necrotrophic lifestyle. Necrotrophic pathogens evolved effectors that actually induce cell death. An elegant example is provided by the *Cochliobolus victoriae* effector victorin that binds to thioredoxins including TRXh5, which is required for redox control of the transcriptional immune regulator NPR1. TRXh5 binding activates the NB-LRR-type immune receptor LOV1, facilitating necrotrophic exploitation of host cell death by *C. victoriae*[29].

In conclusion, although information for the vast majority of pathogen effectors, particularly of filamentous pathogens, is still lacking, effector molecules are highly versatile. Clearly, recently uncovered functions revealed that virulence effectors, despite the finding that they converge onto pivotal elements of the plant immune system[30], can deregulate any step of immunity in any cellular compartment (Figure 1, Table 1).

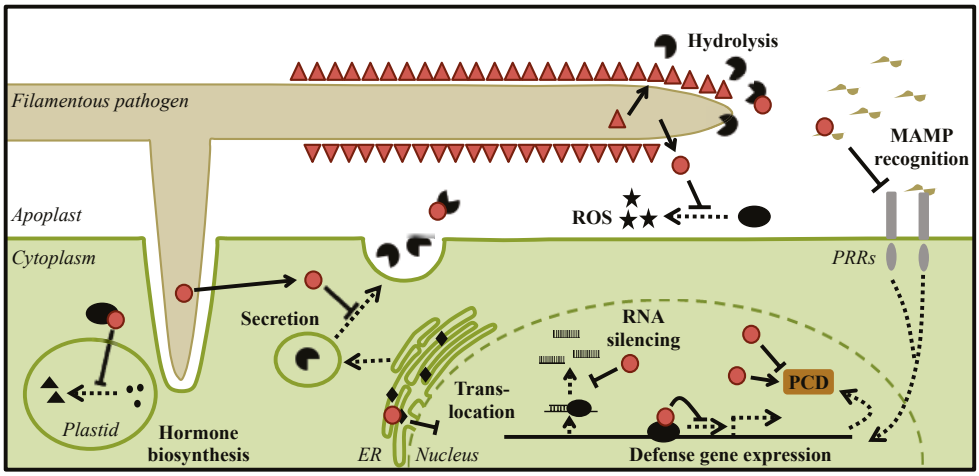

FIGURE 1 | Filamentous pathogen effectors deregulate host immunity in various host subcellular compartments. Pathogens secrete effectors (red symbols) to deregulate plant immunity (see text for details). Whereas one group of effectors (red circles) interacts with host targets that act in immunity (black shapes), another group of effectors (red triangles) acts in self-defense to protect the pathogen from host-derived antimicrobials.

Endophytes and mutualists use effectors to suppress host immunity too

Like pathogens, commensalistic endophytes and mutualists develop intimate host plant associations. During initiation of such symbioses, PRRs continue to perceive MAMPs. Consequently, similar to pathogens, endophytes and mutualists are recipients of immune responses. However, the precise role and fate of host immunity in the establishment of symbiosis has remained enigmatic.

The root endophyte *Serendipita indica* has a wide host range and induces enhanced growth and stress resistance in colonized hosts. Rather than evading host detection, the fungus actively suppresses immunity[31]. During early biotrophic growth at the onset of symbiosis, about 10% of the transcriptome encodes putative effector proteins[32]. At later growth stages the fungus requires host cell death for further colonization, thus resembling hemibiotrophic pathogens such as *Zymoseptoria tritici* and *M. oryzae*. Like *C. fulvum*, these latter species utilize LysM effectors to suppress immune responses[3,5,6]. *S. indica* carries an expanded LysM domain-containing effector repertoire that may similarly act in immune suppression[32].

Effector-like proteins are also encoded by genomes of other mutualists[33-35]. The ectomycorrhiza *Laccaria bicolor* genome encodes hundreds of small, secreted proteins, several of which are only expressed in symbiotic tissues. Of these, MiSSP7 was shown to translocate to the nucleus of poplar host cells to stabilize the JAZ6 protein and repress

jasmonate signaling[34,36]. Likewise, the ectomycorrhiza *Tuber melanosporum* expresses 125 cysteine-rich small secreted proteins, including a LysM effector, which are highly upregulated during symbiosis[35].

TABLE 1 | Effectors of filamentous plant-associated microbes for which molecular virulence targets were identified

Effector	Origin	Target	Function	Reference
BEC4	*Blumeria graminis* f.sp. *hordei*	ARF-GAP proteins	Interference with host vesicle trafficking	41
Avr2	*Cladosporium fulvum*	Cysteine proteases	Cysteine protease inhibition	10,42
Avr4	*Cladosporium fulvum*	Chitin	Hyphal protection	43
Ecp6	*Cladosporium fulvum*	Chitin	Perturbation chitin-triggered immunity	3
CfTom1	*Cladosporium fulvum*	α-tomatine	Detoxification	44
Victorin	*Cochliobolus victoria*	TRX-h5	Induction of LOV1-mediated cell death	29
SP7	*Rhizophagus irregularis*	ERF19	Deregulation of host gene expression	40
HaRxL44	*Hyaloperonospora arabidopsidis*	MED19a	Interference with SA-triggered immunity	23
MiSSP7	*Laccaria bicolor*	JAZ6	Deregulation of host gene expression	33
AvrPiz-t	*Magnaporthe oryzae*	RING E3 ubiquitin ligase APIP6	Suppression of MAMP-triggered immunity	20
Slp1	*Magnaporthe oryzae*	Chitin	Perturbation chitin-triggered immunity	6
MfAvr4	*Pseudocercospora fijiensis*	Chitin	Hyphal protection	45
Mg1LysM	*Zymoseptoria tritici*	Chitin	Hyphal protection	5
Mg3LysM	*Zymoseptoria tritici*	Chitin	Perturbation chitin-triggered immunity	5
Avr3a	*Phytophthora infestans*	CMPG1	E3 ligase stabilization	27
Avrblb2	*Phytophthora infestans*	C14 protease	Suppression of protease secretion	14
EPI1	*Phytophthora infestans*	Serine proteases	Inhibition of serine proteases	46
EPI10	*Phytophthora infestans*	Serine proteases	Inhibition of serine proteases	47
EPIC1	*Phytophthora infestans*	Cysteine proteases	Inhibition of cysteine proteases	11,48
EPIC2B	*Phytophthora infestans*	Cysteine proteases	Inhibition of cysteine proteases	11,48
PexRD2	*Phytophtora infestans*	MAPKKKε	Suppression of host cell death	28
Pi03192	*Phytophthora infestans*	NTP1, NTP2	Suppression of transcription factor relocation	24
GIP1	*Phytophthora sojae*	β-1,3-glucanases	Glucanase inhibition	49
RTP1p	*Uromyces fabae/U. striatus*	Proteases	Protease inhibition	50
Cmu1	*Ustilago maydis*	Cm2	Interference with SA biosynthesis	21
Pep1	*Ustilago maydis*	POX12	Inhibition of peroxidase-mediated ROS production	17
Pit2	*Ustilago maydis*	CP2, CP1A/B, XCP2 proteases	Cysteine protease inhibition	15
Tin2	*Ustilago maydis*	TmTTK1	Control of anthocyanin biosynthesis	22

It was recently shown that arbuscular endomycorrhizal fungi produce lipochito-oligosaccharide mycorrhizal (Myc) factors that stimulate root growth and branching to initiate symbiosis[37]. Similar to endophytes and ectomycorrhiza, arbuscular endomycorrhiza secrete effector-like proteins during symbiotic interactions[38-40]. The genome of *Rhizophagus irregularis* encodes a family of CRN-like proteins that are abundantly found in plant pathogenic *Phytophthora* spp.[39]. *R. irregularis* was furthermore found to encode an effector that interacts with the pathogenesis-related ethylene-responsive transcription factor 19 (ERF19) in the host nucleus to promote mycorrhization, potentially by counteracting MAMP-induced host defense responses that are regulated by ERF19[40].

Collectively, these findings suggest that symbiotic associations that include endophytism, mutualism and parasitism form a continuum in which effectors play essential roles (Table 1).

Effectors act in self-defense and competition

The ability to establish symbiosis evolved multiple times in microbes, presumably from saprotrophism, and many plant pathogens still display saprotrophic life stages. Saprotrophs generally reside within the soil where they feed on decaying organic matter in the presence of a rich microbiota. In this environment, microbial competition as well as co-operation occurs (Figure 2). Threats are posed by (myco)parasites and competitors that produce antibiotics with specific or broad-spectrum activities. Consequently, microbes require molecules for self-defense and interaction with other microbiome partners.

Similar to infected plants, many mycoparasites secrete hydrolytic enzymes including proteases, chitinases and glucanases to target fungal cell walls. Presumably, chitin-binding effectors that protect hyphal cell walls against plant-derived chitinases similarly protect against mycoparasite-derived chitinases, which may explain abundant LysM effector catalogs of non-pathogenic fungi[51,52]. As LysM domains occur in peptidoglycan-binding proteins of various origins, LysM effector homologs that bind non-chitin substrates likely occur. Indeed, a plant pathogen LysM effector that binds bacterial cell walls was characterized (Kombrink and Thomma, unpublished data), potentially implicating this effector in bacterial competition or protection against bacterial mycoparasites. Genome analyses furthermore revealed that saprotrophic species encode abundant catalogs of small, secreted proteins that resemble pathogen effector catalogs[52-55]. Although these potential effectors are poorly studied, one such effector, CipC, was implicated in competition with bacteria in *Aspergillus* spp.[55,56]. The genome of the ubiquitous saprophyte and opportunistic mammalian pathogen *A. fumigatus* encodes several effector proteins[57]. However, since the vast majority of fungi that cause disease in animals are soil saprophytes that opportunistically infect their hosts, to which they are not highly adapted, it has been speculated that infection does not rely on the activity of effectors[58]. Rather, their effectors

are thought to be required for saprophytic survival[58]. Nevertheless, effectors that evolved to enable saprophytic survival may be co-opted for opportunistic infection as well.

Likely, competition between plant-associated microbes also occurs within hosts, although perhaps to a lesser extent than in soil due to reduced species diversity. Indeed, the second most abundantly *in planta*-expressed gene of the fungal endophyte *Epichloë festucae* encodes a secreted antifungal protein[59]. Thus, effector homologs may play crucial roles in microbial competition in a broad spectrum of environments.

Do pathogens shape local microbiomes?

For various types of multicellular organisms it is increasingly recognized that their microbiome, i.e. the community of microbes that thrives in, on, or immediately near the organism, greatly influences its performance[60]. For plants, it has been particularly well documented that the rhizosphere microbiota affects plant growth and stress tolerance. In addition, the importance of the phyllosphere microbiota is increasingly recognized[61]. These microbiota comprise members that provide direct as well as indirect pathogen protection through antibiosis and induced immunity, respectively. Whereas soil types have a major impact on root inhabiting bacterial community compositions on Arabidopsis, host genotypes were reported to only have a minor impact[62,63]. In contrast, different Arabidopsis accessions were found to harbor different phyllosphere communities and several host genetic mutations were found to perturb the microbiota composition, demonstrating that host genetic factors shape the associated microbiota[64]. It is less clear, however, whether plants evolved to actively recruit phyllosphere communities. Potentially, plants recruit founder species that further shape local microbiomes through inter-microbe interactions[61]. Such interactions may require effectors. Considering that plant factors control the composition of the microbiota, microbiome members may utilize effectors to modulate hosts and control competitors indirectly. Additionally, manipulation of host metabolism could even establish microbial cooperation (Figure 2). Although not immediately addressing inter-microbial interactions, an insect-transmitted phytoplasma was recently shown to utilize an effector to alter floral development of host plants, converting them into vegetative tissues that attract leafhopper vectors[65]. This represents a striking example of the exploitation of effector activity to influence compositions of the local biome. Similarly, the rust fungus *Puccinia monoica* induces floral mimicry in the host *Boechera stricta* to enhance its reproduction and spore dispersal by insects[66].

Considering the importance of the microbiome for the ability of plants to withstand pathogen infection, it is conceivable that pathogens evolved to affect host microbiomes, possibly through effector activities (Figure 2).

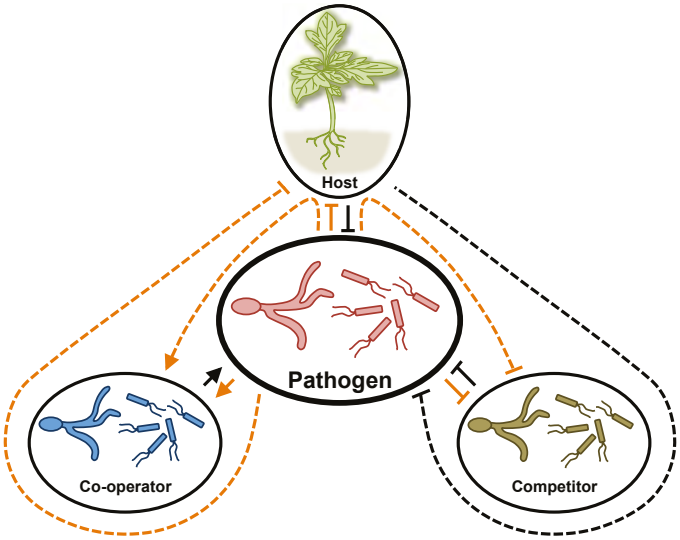

FIGURE 2 | How pathogens influence the local biota by exploiting effector activities. The interaction between microbial pathogens and plant hosts occurs in environments that contain additional microbiome partners that can negatively (competition) or positively (co-operation) impact the pathogen as well as the host. Consequently, the pathogen and host may target each other directly (solid lines) as well as indirectly (dotted lines). Likely, pathogens exploit effector activities (orange lines) to not only directly modulate their hosts, but also to influence the local microbiota that can impact the outcome of the interaction with their hosts.

Different mechanisms drive evolution of effector repertoires

Mechanisms underlying genome plasticity and evolution have been intensely studied, especially for plant pathogens. As genomes are structured and not just a random sequence of genes, effector genes are often found in dynamic genomic compartments, such as gene-sparse regions, subtelomeric regions or conditionally dispensable (pathogenicity) chromosomes[67]. For example, effector localization in gene-sparse regions was recorded for the endophyte *S. indica*[32], while in the saprophyte *N. crassa* genes encoding small-secreted proteins are found in subtelomeric regions[53]. Genetic plasticity in such compartments is governed by diverse mechanisms including recombination and activity of transposable elements. A direct implication of genomic rearrangement in the evolution of fungal aggressiveness was shown for the vascular wilt fungus *Verticillium dahliae*, leading to the emergence of lineage-specific regions that are enriched for virulence effectors[68]. High genetic variability in effector genes enables rapid evolutionary processes. The importance of dynamic genome compartments for accelerated gene evolution was underlined in the specialization of *P. infestans* after the host jump that separated this species from related species. Uneven evolutionary rates across the genome occur, with *in planta*-induced genes residing in fast evolving compartments[69]. In turn, effector specialization can lead

to diversification and speciation in pathogen lineages[13]. In this manner, effectors can determine microbial niches. Moreover, composition of effector catalogs can dictate microbial lifestyles. For example, the leaf epiphyte and antagonist of powdery mildews *Pseudozyme flucculosa* lost its ability to parasitize plants like its smut fungi relatives due to loss of virulence effectors[70]. However, the biocontrol agent has acquired other effectors that are not found in the smut relatives that may have shaped its current lifestyle[70]. These findings suggest that effector catalogs evolve via different mechanisms and that their composition influences a microbe's lifestyle in a given environment.

Experimental way forward

The interaction between pathogenic (filamentous) microbes and the organisms they encounter in their niches, either while colonizing the host or during free-living stages in the environment, is poorly understood. An extensive characterization of the complex microbial communities in such niches may lead to a better understanding of the interactions that take place beyond the direct interaction between pathogen and host. Detailed transcriptome analyses may lead to the identification of particular triggers of effector gene expression derived from microbial co-inhabitants, and may hint towards functions in inter-microbial interactions[71,72] that can subsequently be tested for in targeted analysis to reveal components that either promote or inhibit other microbes[52].

Conclusions

Although a paradigm in plant pathology dictates that existence of disease requires the interaction of a virulent pathogen with a susceptible host in a favorable environment, plant-microbe interactions are mostly studied as one-on-one relationships. However, in addition to host immune responses, pathogenic microbes continuously encounter other microbes that include competitors and mycoparasites that need to be dealt with simultaneously. Importantly, findings for pathogenic microbes can be extrapolated to other types of symbioses as well. After all, irrespective of the type of symbiosis, the interest of the microbial partner is merely to exploit the host for nutrition and shelter. This may also explain the thin line that is regularly observed between the different types of symbioses[32,33,73,74]. In all types of symbioses, the microbial partner needs to suppress host immune responses and ward off microbial antagonists. Using effectors as probes, further critical processes in host colonization will be uncovered, leading to enhanced understanding of the biology of microbes that aim to establish symbioses.

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Chapter 3

The fungal effector protein Ecp6 interferes with chitin perception in the model plant *Arabidopsis thaliana*

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Abstract

Fungal microbes utilize effector proteins to suppress chitin-triggered immunity during plant colonization. The tomato leaf mold fungus *Cladosporium fulvum* secretes the lysin motif (LysM)-containing effector protein Ecp6. Ecp6 has the capacity to outcompete plant chitin receptors through cooperative high affinity binding of chitin substrates between two of its three LysM domains. Additionally, the singular second LysM domain (LysM2) can perturb chitin responses in tomato through a yet unknown mechanism. Due to its relatively low affinity for chitin, it has been hypothesized that LysM2 interferes with chitin receptor dimerization required for the activation of immunity. To further investigate Ecp6 functionality, we utilized the model plant *Arabidopsis thaliana* for which chitin perception has been intensely studied. Here, we show that Ecp6 suppresses chitin-mediated immune responses in *A. thaliana*. While Ecp6 does not suppress phosphorylation of the LysM receptor kinase AtCERK1, it affects internalization of the LysM receptor kinase AtLYK5 in a ligand-dependent manner. These findings suggest that, in *A. thaliana*, Ecp6 affects chitin perception by targeting chitin receptors through a yet unknown mechanism.

Introduction

Chitin is one of the most abundant carbohydrates in nature, and represents a major component of fungal cell walls. During early stages of host colonization, invasive hyphae of filamentous microbes encounter host-derived hydrolytic enzymes, such as chitinases, that release chitin oligosaccharides from fungal cell walls[1]. Recognition of chitin oligosaccharides by host receptors prompts the secretion of antimicrobial compounds and toxins, as well as the release of additional cell wall degrading enzymes such as endochitinases that hydrolyze fungal hyphae. These chitin-dependent immune responses are detrimental to fungal growth and may eventually halt host invasion[2,3].

In rice (*Oryza sativa*), chitin recognition is mediated by the lysin motif (LysM)-containing Chitin Elicitor Binding Protein (OsCEBiP), which lacks an intracellular kinase domain[4]. OsCEBiP dimerizes upon ligand binding and forms a tetrameric receptor complex with Chitin Elicitor Receptor Kinase 1 (OsCERK1) to initiate chitin signalling[5,6]. As in rice, *Arabidopsis thaliana* (hereafter Arabidopsis) CERK1 is required for chitin signalling[7,8]. However, unlike OsCERK1, AtCERK1 directly binds long chain chitin fragments via its second LysM domain with a relatively low affinity[9-11]. In addition to AtCERK1, the LysM receptor kinases AtLYK4 and AtLYK5, both of which lack active kinase domains, were identified as chitin-binding proteins[10]. AtLYK5 was shown to interact with AtCERK1 in a ligand-dependent manner, required for AtCERK1 dimerization and phosphorylation[12]. Due to its higher affinity for longer chain chitin oligomers, AtLYK5 was proposed as the primary chitin receptor in Arabidopsis. Remarkably, despite its high affinity for chitin oligomers[13], the Arabidopsis orthologue of OsCEBiP, LysM domain protein 2 (AtLYM2), is not involved in AtCERK1-mediated chitin responses but rather modulates plasmodesmata conductivity in a AtCERK1-independent manner[14].

To overcome or bypass chitin-triggered immune responses, filamentous microbes employ various strategies, including cell wall modifications and the secretion of LysM-containing effector proteins that suppress chitin-triggered immune responses[15-17]. Moreover, effector proteins can shield cell wall chitin, thereby preventing hydrolysis by plant chitinases. For example, the tomato leaf mold fungus *Cladosporium fulvum* produces avirulence protein 4 (Avr4), which binds chitin through its invertebrate chitin-binding domain (CBM14)[18,19]. While Avr4 protects invasive hyphae, it is not able to perturb chitin-induced immune responses.

In addition to Avr4, *C. fulvum* secretes the LysM effector protein Ecp6. Ecp6 compromises chitin-induced immune responses by chitin binding with ultra-high (pM) affinity through intramolecular LysM domain dimerization, thereby outcompeting host receptors[20]. Interestingly, the singular LysM2 domain of Ecp6, which is not involved in intramolecular chitin binding, retains the capacity to perturb chitin-induced immune responses[20,21]. Due to its lower chitin affinity, it is unlikely that LysM2 deregulates chitin-triggered

immunity by chitin fragment sequestration[22]. Therefore, it has been hypothesized that LysM2 may interfere with the chitin-induced host receptor dimerization that is required for the activation of immune signalling[3,20]. Since the mechanisms underlying chitin recognition have not been identified in the *C. fulvum* host plant tomato, we made use of the Arabidopsis model system to study the potential role of Ecp6 in the perturbation of chitin receptor complex activation.

Results and Discussion

When applied to leaf discs of Arabidopsis ecotypes Col-0 or WS-4, polymeric chitin triggers the production of reactive oxygen species (ROS). ROS production can be detected and reaches its maximum at approximately 15-20 minutes following treatment (Fig. 1). This response is abolished in the *cerk1-2* (Col-0) and *cerk1-3* (WS-4) mutants (Fig. 1). Intriguingly, despite being derived from a fungal pathogen that has tomato as its sole host, addition of *Pichia pastoris*-produced Ecp6 protein prevents chitin-triggered ROS production in Arabidopsis wild type ecotypes (Fig 1). This is in accordance with previous results, which showed suppression of ROS generation by Ecp6 upon chitin treatment in tomato[21]. Ecp6 ability to perturb chitin-induced immune responses in tomato is mediated by its LysM2 domain, since *P. pastoris*-produced Ecp6 mutated in the putative chitin-binding site of LysM2 ($Ecp6^{T95R}$) no longer suppresses chitin responses[20]. Surprisingly, however, simultaneous application of polymeric chitin and $Ecp6^{T95R}$ effectively reduces the generation of ROS in Arabidopsis, similar to wild type Ecp6 protein (Fig. 1). These results suggest that Ecp6 is functional in Arabidopsis but that its mode of action may differ between plant species. Alternatively, since Arabidopsis is less sensitive to chitin than tomato[23], Ecp6 ability to sequester chitin fragments through its high-affinity binding site may be sufficient to suppress chitin-induced immune responses in Arabidopsis. However, we cannot exclude the possibility that differences between batches of purified protein produced in *P. pastoris* result in variations of Ecp6 activity irrespective of the plant species tested.

Chitin receptors interact in a ligand-dependent manner to activate downstream signalling[24]. In a previous report, the chitin-triggered interaction between AtCERK1 and AtLYK5 was shown following their production in Arabidopsis protoplasts[12]. To be able to test whether Ecp6 interferes with this complex formation, we intended to confirm the occurrence of an interaction between AtLYK5 and AtCERK1 in transgenic Arabidopsis lines producing mCitrine-tagged AtLYK5. To this end, we vacuum-infiltrated Arabidopsis leaves with a chitin suspension or water as negative control, and immunopurified AtLYK5-mCitrine from crude protein extracts. Immunopurification of AtLYK5-mCitrine following chitin treatment did not result in co-purification of AtCERK1 (Fig. 2). In a further attempt to demonstrate an interaction between AtLYK5 and AtCERK1 using Arabidopsis leaf tissue, we infiltrated the cross-linking agent ethylene glycol bis(succinimidyl succinate) (EGS) 30

min after chitin treatment. However, we failed to observe co-purification of AtCERK1 with AtLYK5-mCitrine even following cross-linking (Fig. 2). It has been hypothesized that the interaction between AtCERK1 and AtLYK5 is transient upon perception of chitin[25]. Thus, the use of whole Arabidopsis leaves may be inappropriate to visualize this transient interaction. Consequently, based on the results presented here, we were not able to test a possible inhibitory effect on chitin receptor dimerization by Ecp6.

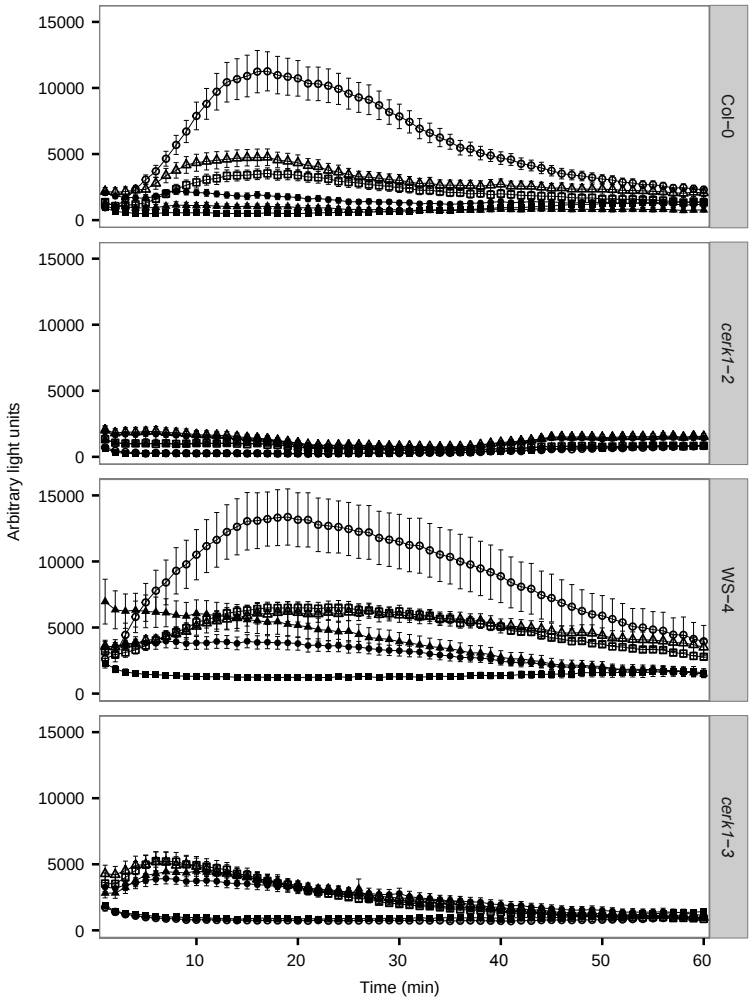

FIGURE 1 | Ecp6 suppresses ROS production in Arabidopsis. Generation of ROS was measured using luminol-based chemiluminescence for 60 min following treatment of Arabidopsis leaf discs with 10 µg/mL polymeric chitin (○), 10 µM Ecp6 (●), 10 µM Ecp6^{T95R} (▲), Ecp6+chitin (△), Ecp6^{T95R}+chitin (□), or water (■) as negative control. Plotted are chemiluminescence averages of *n*=8 leaf discs ± S.E.. The figure is representative of at least three independent experiments.

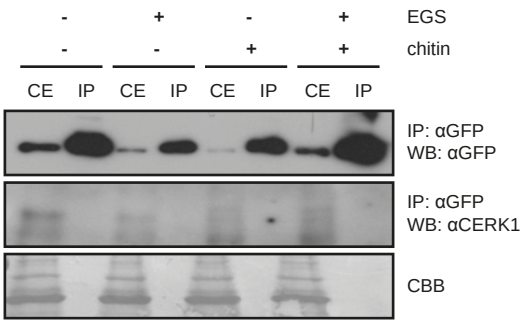

FIGURE 2 | Treatment with a cross-linking agent is not sufficient to demonstrate chitin receptor interaction in stably transformed Arabidopsis lines. Western blot (WB) showing immunopurification (IP) of AtLYK5-mCitrine from crude leaf extracts (CE) of transgenic Arabidopsis treated with water or 10 µg/mL polymeric chitin. Thirty min after elicitation, leaves were treated with the cross-linking agent ethylene glycol bis(succinimidyl succinate) (EGS). Membranes were stained with Coomassie brilliant blue (CBB) to confirm equal loading. The experiment was repeated twice with similar results.

Chitin perception results in the phosphorylation of AtCERK1 and AtLYK5, and AtCERK1 phosphorylation is required for the activation of signal transduction[25]. Phosphorylation can be detected as a band shift in total protein extracts on SDS polyacrylamide gels and serves as a proxy for receptor complex activation. AtCERK1 shifted on gel after chitin treatment in Col-0 and much less in *lyk5-2* mutants (Fig. 3a), confirming AtLYK5 involvement. Moreover, immunoprecipitation with chitin beads resulted in the recovery of less AtCERK1 protein from crude extracts following chitin infiltration when compared with water treatment (Fig. 3a). Several receptors, including the leucine-rich repeat (LRR) receptor kinase FLAGELLIN SENSING 2 (FLS2) and the LRR receptor protein Cf4, are internalized and targeted for degradation upon ligand perception[26-29]. Consequently, the overall abundance of FLS2 and Cf4 decreases following treatment with their ligands flg22 and Avr4, respectively[27,30,31]. However, AtCERK1 can be continuously detected at the plasma membrane and no vesicle formation is observed following chitin treatment[25]. Thus, the reduced recovery of AtCERK1 with chitin beads is likely due to the lower availability of chitin-free AtCERK1 molecules following chitin treatment. We then tested whether AtCERK1 phosphorylation is diminished following chitin treatment in the presence of Ecp6. While the lower band for AtCERK1, which represents AtCERK1 in absence of chitin-triggered phosphorylation[10], appeared to be stronger when leaves were treated both with Ecp6 and chitin when compared to treatment with chitin only, AtCERK1 phosphorylation was not suppressed (Fig. 3b). Interestingly, however, immunoprecipitation with chitin beads following total protein extraction resulted in increased recovery of AtCERK1 receptor protein in the presence of Ecp6 and chitin. This suggests that Ecp6 (indirectly) affects AtCERK1 chitin binding. Possibly, Ecp6 sequesters chitin molecules, leaving fewer molecules for AtCERK1, and thus augmenting the amount of AtCERK1 chitin binding sites that remain accessible to immunoprecipitation with chitin beads. Similar results were obtained after co-treatment with chitin and Ecp6^{T95R} (Fig. 3b).

Since Ecp6 interferes with chitin-dependent defense responses in Arabidopsis, but does not markedly affect AtCERK1 phosphorylation, we tested whether Ecp6 can interfere with AtLYK5 internalization. AtLYK5 is internalized into endosomes starting approximately 20

min and peaking around 60 min after chitin treatment[25]. In addition to polymeric chitin, chitin heptamers (GN7) and octamers (GN8) are able to induce AtLYK5 endocytosis at 10 µM final concentrations (Fig. 4). Surprisingly, although co-infiltration of chitin oligomers with Ecp6 at equimolar concentrations did not change the number of observed vesicles, the vesicles became more prominent (Fig. 4). In contrast, treatment with Ecp6 in the presence of polymeric chitin resulted in the formation of fewer vesicles but did not affect their size (Fig. 4). These observations suggest that Ecp6 affects AtLYK5 internalization in a ligand-dependent manner.

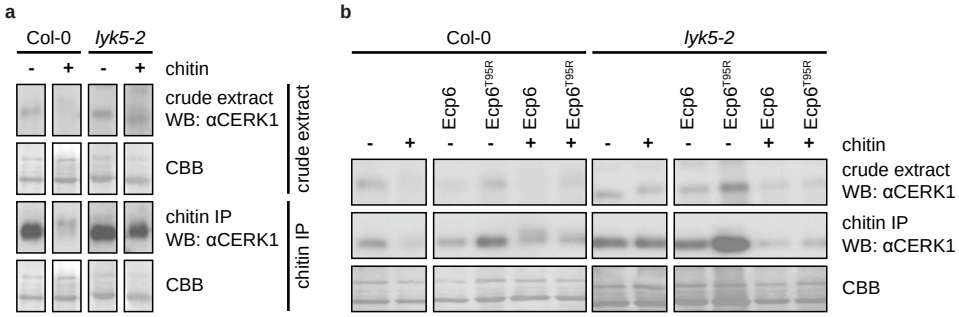

FIGURE 3 | Ecp6 does not prevent AtCERK1 phosphorylation. AtCERK1 was detected using α-CERK1 antibody in crude protein extracts from Col-0 or *lyk5-2* leaf tissue after treatment with water or 10 µg/mL polymeric chitin in absence (**a**) or presence (**b**) of Ecp6 or Ecp6^{T95R} (3 µM). Chitin binding of AtCERK1 was confirmed in pull-downs with chitin beads. Membranes were stained with Coomassie brilliant blue (CBB) after Western blotting to confirm equal loading. The results shown are representative of three independent experiments.

In summary, we show that Ecp6 perturbs chitin-triggered immune responses in Arabidopsis. However, its role and in particular the contribution of the singular LysM2 domain to Arabidopsis chitin receptor inhibition remain elusive. Importantly, Ecp6 is produced by the tomato pathogen *C. fulvum* and a translation into the tomato system is required to fully unravel Ecp6 functionality.

Materials

Plant materials

Plants were grown under short day conditions (8 h photoperiod), at 22°C/18°C during day/night cycles, with 65% relative humidity and light intensity approximately 150 µmol/m^{2}s. Plants were used for experiments after 4-6 weeks. *In vitro* seedlings were grown in liquid ½ Murashige and Skoog medium supplemented with 0.5% sucrose for 2 weeks. The Arabidopsis T-DNA lines used in this study were *cerk1*-2 (GABI_096F09)[7], *lyk5*-2 (SALK_131911C)[7,12] and *cerk1-3* (FLAG_GX112, INRA line)[32]. Vesicle formation was observed using transgenic *pLYK5::LYK5-mCitrine*[25].

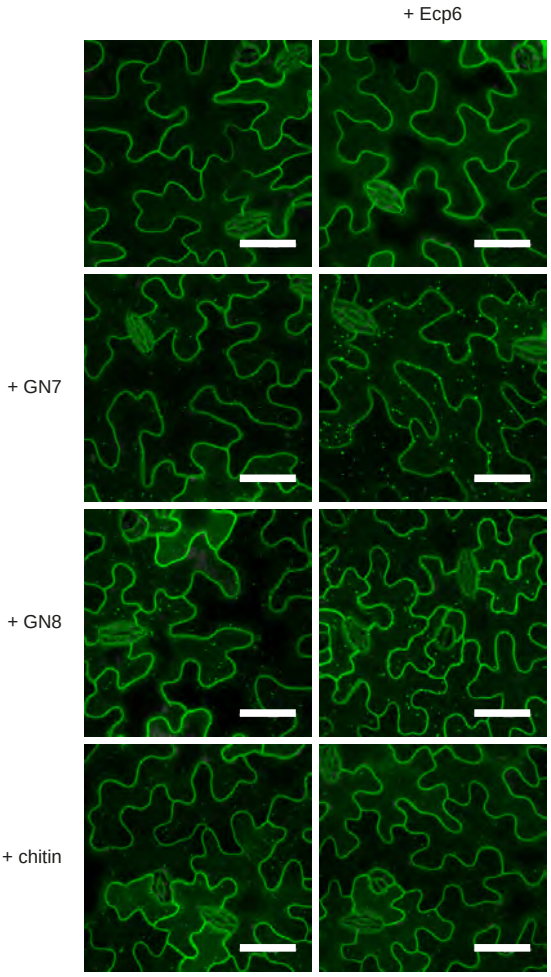

FIGURE 4 | Ecp6 affects chitin-dependent AtLYK5 endocytosis. Leaves of Col-0 plants stably producing AtLYK5-mCitrine were infiltrated with water, 10 µM GN7, 10 µM GN8, or 10 µg/mL polymeric chitin with or without 10 µM Ecp6. Internalization of AtLYK5-mCitrine was detected 60 min after treatment. Shown are maximum projections of 8 focal planes. Size bar = 30 µm.

Production and purification of recombinant effector proteins

Ecp6 was expressed in *Pichia pastoris* strain GS115 and purified as described previously[21,33]. For treatment of plant tissue, recombinant effector proteins were desalted over PD10 columns (GE Life Sciences) according to manufacturer's instructions.

Chemicals and plant treatments

For induction of vesicles, chitin (Sigma-Aldrich), and chitooligomers GN7 and GN8 (IsoSep) were vacuum-infiltrated into leaf pieces in absence or presence of 10 µM Ecp6. Polymeric chitin was used at a concentration of 10 µg/mL and oligomers at 10 µM. The incubation time was 60 min. For assaying phosphorylation of CERK1 100 µg/mL chitin was vacuum-infiltrated into detached leaves and incubated for 10 min. To test the suppression of phosphorylation by Ecp6 10 µg/mL chitin was infiltrated in absence or presence of 3 µM Ecp6 or Ecp6^{T95R}.

Oxidative burst assay

Oxidative burst measurements were performed as previously described[32] with the following modifications: Water was replaced with 100 µL of a solution containing 100 µM L-012 substrate and 20 µg/mL horseradish peroxidase. ROS was elicited with 10 µg/mL polymeric chitin in absence or presence of 10 µM recombinant effector protein. Eight leaf discs were used for each condition. Luminescence was measured over 60 min using an Infinite® M200 multimode reader (TECAN) in combination with the i.control software package (Version 1.6).

Protein extraction, affinity purification and immunoblotting

Receptor cross-linking was carried out *in vivo* by vacuum-infiltration with ethylene glycol bis(succinimidyl succinate) (EGS) of water- or chitin-treated leaves. Total protein extractions and chitin pull-downs were performed as described previously[10]. For receptor co-immunoprecipitations total extracts were mixed with 20 µL magnetic chitin beads (New England Biolabs) and incubated at 4°C, 10 rpm for 1 hr. Beads were washed three times with 1 mL TBS-T. Affinity purified proteins were eluted in 40 µL 2x SDS buffer. Proteins were separated by 8 or 10% SDS-PAGE and blotted to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were probed with anti-CERK1[10,32] or HRP-linked anti-GFP (ChromoTek). A goat-anti-rabbit alkaline phosphatase conjugate (Sigma-Aldrich) was used as secondary antibody for αCERK1. Reactions were detected using the ImmunStar AP substrate (Bio-Rad) or with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific).

Confocal microscopy

Confocal microscopy was performed on a Leica TCS SP5 system (Leica Microsystems, Wetzlar, Germany) equipped with an argon laser and HyD hybrid detectors. mCitrine was excited at 514 nm, and emission was recorded between 525 and 560 nm. Chlorophyll autofluorescence was detected between 740 and 770 nm. Images were processed using the Leica LAS AF (Version 2.7.2.) software package. All the phenotypes were observed in at least two independent transgenic Arabidopsis lines.

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Author contributions

H.R. and B.P.H.J.T. designed and conceived the experiments. H.R., E.K.P., J.E. and V.L. performed the experiments and analyzed the data. H.R. and B.H.P.J.T. wrote the manuscript.

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Chapter 4

A LysM receptor kinase mediates chitin-triggered defense responses in tomato

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Abstract

Plants detect the presence of potential fungal pathogens by sensing the conserved cell wall component chitin. To date, all plant chitin receptors identified belong to the class of membrane-exposed lysin motif (LysM)-containing receptor proteins. Here, we identify two chitin-binding LysM receptors of tomato using an affinity purification approach. Based on their phylogenetic relationship to well-characterized chitin receptors of rice and *Arabidopsis thaliana*, the tomato receptor candidates were named SlCEBiP and SlLYK4, respectively. Silencing of *SlLYK4*, but not of *SlCEBiP*, resulted in compromised tomato responsiveness to chitin. These results suggest that SlLYK4 is a major component of the receptor complex in tomato that activates the canonical chitin signal transduction pathway.

Introduction

Plants detect the presence of invading microbes through cell surface-localized receptor molecules, which recognize either microbe-derived or modified-self molecules that indicate invasion[1]. This recognition leads to a series of cellular events that either promote or restrict microbial colonization[2]. The activation of immune responses, which include the generation of extracellular reactive oxygen species (ROS), an increase in cytosolic calcium concentrations, the activation of mitogen-activated protein (MAP) kinase and calcium-dependent protein kinase (CDPK) cascades as well as changes in gene expression, protect the plant from invasion by potential pathogens[3].

Bacterial molecules that are recognized by plant receptor proteins include flagellin, elongation factor Tu (EF-Tu), peptidoglycan or lipopolysaccharide[3]. In contrast, filamentous microbes are generally perceived due to the presence of their major cell wall components chitin or β-glucan[4,5]. Chitin consists of β-1,4-linked polymers of *N*-acetylglucosamine (GlcNAc) with varying degrees of polymerization. While longer chitin oligomers are potent inducers of immune responses[6,7], chitin tetra- and heptamers have been implicated in mutualistic symbiosis[8,9]. Interestingly, bacterial peptidoglycan as well as lipo-chito-oligosaccharides (LCOs) produced by beneficial rhizobacteria and arbuscular mycorrhizal (AM) fungi, respectively, are chitin derivatives.

Until now, the receptor molecules that have been implicated in the recognition of GlcNAc-containing molecules belong to the group of lysin motif (LysM) receptor proteins, which are classified as receptor kinases (RKs) or receptor proteins (RPs)[10]. The extracellular portion of LysM-RKs, which contains the LysM domains, is coupled to an intracellular kinase domain via a single pass transmembrane domain. Based on differences in their kinase domain features, LysM-RKs are further divided into LYKs and LYRs[10,11]. In contrast to LYKs, the kinase domains of LYRs lack most structural components required for kinase activity. LysM-RPs (also referred to as LYPs) are membrane-bound via a glycosyl phosphatidylinositol (GPI) anchor and lack an intracellular kinase domain for activation of downstream signaling.

Signal transduction requires the presence of receptor complexes containing LYK receptor molecules, which relay the signal via their intracellular kinase domain. For example, in *Arabidopsis thaliana* (hereafter referred to as Arabidopsis), the LYR AtLYK5 binds chitin with high affinity and forms a heteromeric complex with the receptor kinase CERK1 to initiate chitin signalling[12]. While AtLYK5 has been proposed as the main chitin receptor in Arabidopsis, its paralogue AtLYK4 has also been implicated in chitin recognition and only *lyk4lyk5* double mutants show a complete loss of chitin responsiveness[12-14]. As in Arabidopsis, rice (*Oryza sativa*) CERK1 is required for chitin signaling following its ligand-induced association with the LYP receptor OsCEBiP[15-18]. Interestingly, one of the Arabidopsis CEBiP orthologues, AtLYM2 controls the chitin-induced flux across plasmodesmata and resistance to fungal

pathogens independent of AtCERK1[19,20]. In addition to chitin signaling, CERK1 from both rice and Arabidopsis has been shown to be required for peptidoglycan-triggered immunity[21-24]. Interestingly, in addition to its role in immunity, OsCERK1 has also been implicated in AM symbiosis that is established upon perception of fungal LCOs[18,25]. These findings suggest that CERK1 likely acts as a co-receptor in different receptor complexes[4]. Similar to fungal LCOs, also bacterial LCOs are recognized by LysM receptor pairs in leguminous plant species during the establishment of symbiosis with rhizobacteria[26-29].

Based on the findings in rice and Arabidopsis, it is tempting to assume that also in tomato LysM receptors are required for the perception of GlcNAc-containing molecules. Indeed, the LYR receptor kinase SlLYK10 has recently been shown to play a role in colonization by the AM fungus *Rhizophagus irregularis*[30]. Indirect evidence suggests that the CERK1 orthologues SlLYK1 and SlLYK13 may be required for tomato immunity to bacterial pathogens as they are targeted by the bacterial effector protein AvrPtoB to suppress plant immune responses[31].

Here we identified two LysM receptors, SlLYK4 and SlCEBiP, with a putative role in chitin perception using a biochemical approach. Virus-induced gene silencing of SlLYK4, but not of SlCEBiP, resulted in reduced chitin responsiveness, suggesting that SlLYK4 mediates chitin-triggered immune responses in tomato.

Results

Mass spectrometry identifies two LysM receptors as candidate chitin receptors

To identify the receptor protein(s) involved in chitin perception in tomato, we isolated microsomal proteins from tomato cv. Heinz 1706 leaf tissue for affinity purification with magnetic chitin beads[32]. Specifically bound proteins were eluted either with chitohexaose ($(GlcNAc)_6$ abbreviated as GN6) or chitosan. In order to elute the remaining non-specifically bound proteins, chitin beads were boiled in sample buffer. Following size-separation by SDS-PAGE, proteins in the range of 50-100 kDa were subjected to trypsin digestion. Subsequent mass spectrometric analysis yielded several unique peptides in both GN6 and chitosan eluents for only two LysM-containing receptors encoded by *Solyc02g089900* and *Solyc01g112080* (Table 1; Supplementary Fig. 1). Unique peptides for the same receptors were also detected in the on-bead fractions, suggesting that the elution with GN6 and chitosan had been incomplete. Moreover, only 0 and 1 unique peptide were found in the supernatant following affinity purification (unbound fraction) for Solyc02g089900 and Solyc01g112080, respectively, demonstrating that the majority of both proteins present in the whole leaf extracts bound to chitin beads (Table 1).

TABLE 1 | Identification of chitin-binding LysM proteins of tomato (*Solanum lycopersicum* Heinz 1706)
Chitin-binding proteins identified from microsomal fractions. Numbers represent unique peptides identified by Sequest and/or Mascot analyses.

Gene ID	Name	Subfamily	ΣCoverage (%)	Size (aa)	MW (kDa)	pI	Unbound	GN_6 eluent	GN_6-treated beads	Chitosan eluent	Chitosan-treated beads
Solyc02g089900	SlLYK4	LYR	17.2	645	70	5.3	0	3	4	4	4
Solyc01g112080	SlCEBiP	LYP	18	345	37	5.4	1	4	4	3	3

Solyc02g089900 codes for a membrane-bound LYR with three surface-exposed LysM domains that is closely related to Arabidopsis LYK4, and is therefore referred to as SlLYK4 (Fig. 1; Fig. 2)[30]. Phylogenetic analysis of full-length LysM receptor protein sequences showed that, in addition to AtLYK4, SlLYK4 forms a clade together with AtLYK5, SlLYK6, SlLYK7, and SlLYK15 on a longer branch, as was shown previously[30]. *Solyc01g112080* encodes a receptor protein with extracellular LysM domains that are membrane-bound via a GPI anchor (Fig. 2). Sequence analysis with the domain prediction algorithm InterPro (http://www.ebi.ac.uk/interpro/) suggested the presence of two LysM domains in SlCEBiP. However, amino acid sequence alignment with its orthologue OsCEBiP suggests that SlCEBiP, like OsCEBiP, *Medicago truncatula* LYM2 and Arabidopsis LYM2, contains three instead of two LysMs (Fig. 1; Fig. 2; Supplementary Fig. 2)[16,33-35]. Considering the role of their orthologues in chitin signaling in Arabidopsis, rice and *M. truncatula*, we further investigated the role of SlLYK4 and SlCEBiP in chitin recognition of tomato.

SlLYK4 and *SlCEBiP* gene expression is induced upon fungal infection

To analyze the expression patterns of the two putative tomato chitin receptor genes, we first examined publicly available transcriptome data (see Methods section for details). The expression levels of both *SlCEBiP* and *SlLYK4* varied greatly between the two tomato cultivars analyzed (Heinz 1706 and Moneymaker) as well as among the various tissues tested (Supplementary Fig. 2). *SlLYK4* is mainly expressed in roots in tomato cv. Moneymaker, whereas transcript accumulation is the highest in mature fruits of tomato cv. Heinz (Supplementary Fig. 2a). In contrast, *SlCEBiP* is highly expressed in root tissue of both cultivars (Supplementary Fig. 2b)[30]. Moreover, in tomato cv. Moneymaker expression values vary little between root, stem and leaf tissue, whereas few *SlCEBiP* transcripts were detected in leaves, flowers and mature fruits of tomato cv. Heinz 1706.

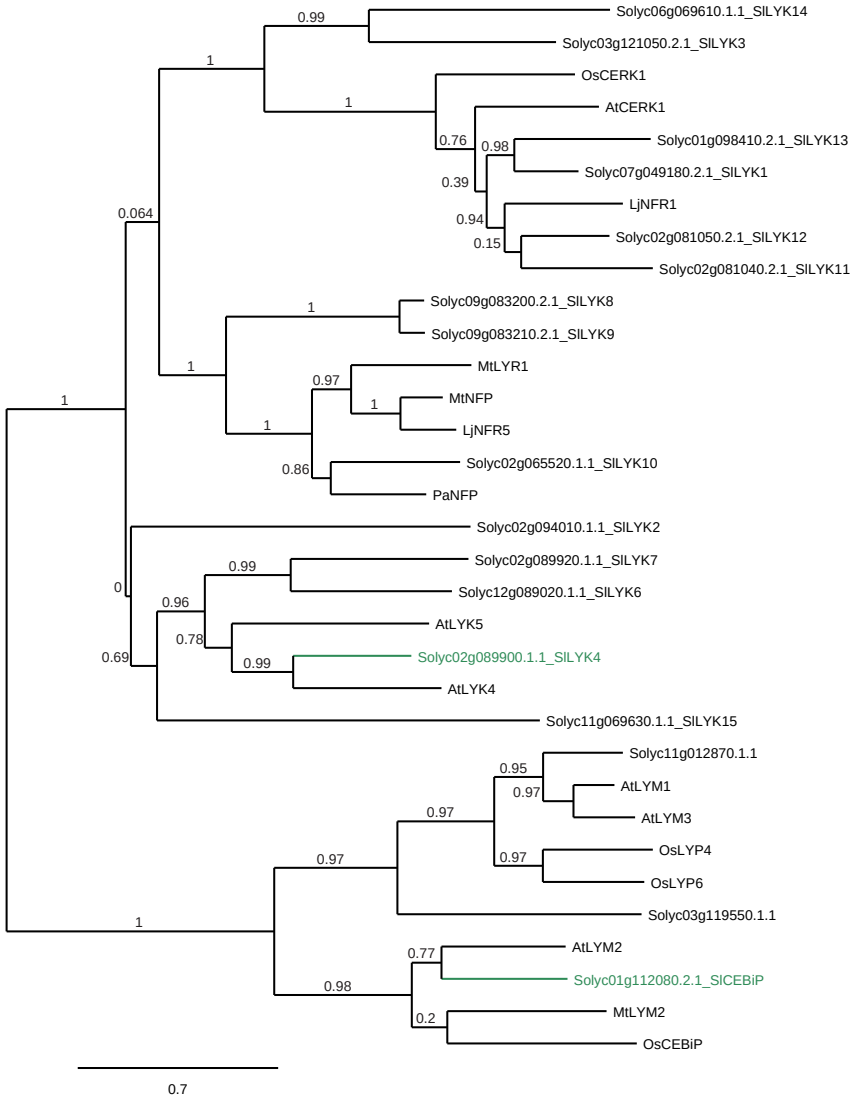

FIGURE 1 | Phylogenetic analysis of LysM receptor proteins. Tomato LysM proteins were selected based on the presence of an extracellular domain containing LysM motifs as well as their predicted localization to the plasma membrane due to the presence of a predicted transmembrane domain or a GPI anchor. Their phylogenetic relationship to LysM receptor proteins of rice, Arabidopsis, *Lotus japonicas*, and *Medicago truncatula* was inferred based on the maximum likelihood method. SlLYK4 and SlCEBiP are highlighted in green.

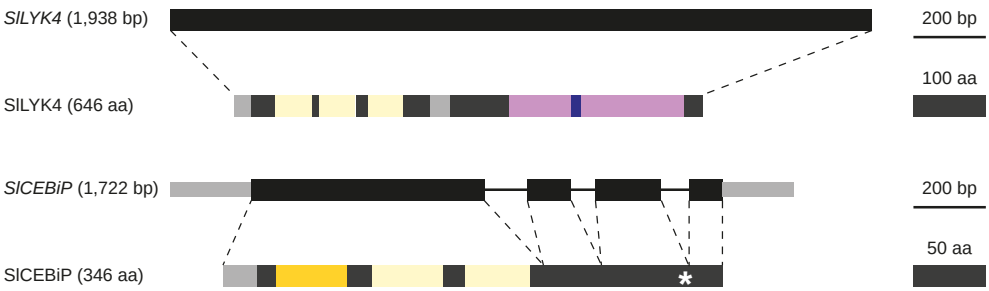

FIGURE 2 | Graphic representation of gene and encoded protein structures of putative tomato chitin receptors. Genes are represented by black boxes (exons), black lines (introns) and grey boxes (5´ and 3´UTR). The protein structures include signal peptides and the transmembrane domain for SlLYK4 (grey boxes), LysM domains predicted by InterPro (yellow), one LysM domain in SlCEBiP inferred from pairwise alignment with OsCEBiP (Supplementary Fig. 2) (orange box), and the intracellular kinase domain with the catalytic loop of SlLYK4 (violet and blue, respectively). The white asterisk marks the predicted site for the attachment of a GPI anchor in SlCEBiP.

Next, changes in *SlLYK4* and *SlCEBiP* gene expression upon challenge with a fungal pathogen were tested. To this end, tomato plants were inoculated with the vascular wilt fungus *Verticillium dahliae* and stem tissue was collected at 4, 8, and 12 days following inoculation. Quantitative RT-PCR analysis showed that transcript accumulation of both genes is enhanced during pathogen infection compared to healthy control plants (Fig. 3). *SlCEBiP* expression levels peak at around 8 days post inoculation (dpi), whereas *SlLYK4* expression is highest at 4 dpi and then gradually decreases. These results suggest that both *SlCEBiP* and *SlLYK4* may be implicated in chitin perception during plant invasion by fungal pathogens.

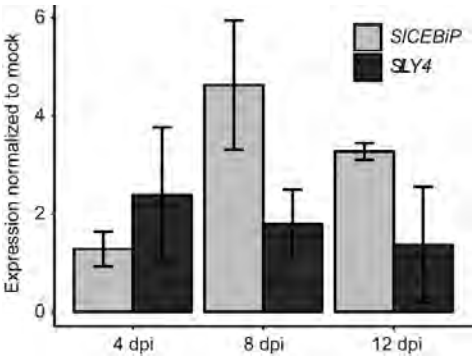

FIGURE 3 | *SlLYK4* and *SlCEBiP* are expressed in tomato stem tissue during *Verticillium* infection. Tomato stem tissue was harvested at 4, 8, and 12 days following inoculation with *V. dahliae*. LysM receptor gene expression levels were determined by qRT-PCR with gene-specific primers and primers targeting the tomato tubulin gene for calibration (Supplementary Table 1). Shown are expression values relative to mock-treated tissue of three biological replicates consisting of pools of three plants from a single experiment (mean ± S.E.). This experiment was performed twice.

Silencing of *SlLYK4* impairs chitin-triggered responses

When perceived by plants, chitin triggers the activation of multiple downstream responses, including the generation of reactive oxygen species (ROS) and changes in gene expression. In order to determine whether *SlLYK4* or *SlCEBiP* contributes to chitin recognition and,

thus, the activation of chitin-triggered immunity in tomato, we generated tobacco rattle virus (TRV)-based constructs to silence both genes separately. Tomato plants were treated with TRV:*SlLYK4*, TRV:*SlCEBiP* or TRV:*GUS* as negative control. Three weeks later, two of the youngest, fully expanded leaves were collected from each plant and tested for their ability to respond to chitin. Silencing efficiency and specificity were confirmed by quantitative RT-PCR in mock (water)-treated samples (Supplementary Fig. 5). As expected, treatment with GN6 resulted in ROS generation in *GUS*-silenced plants within 2.5 min (Fig. 4a). However, silencing of *SlLYK4* greatly impaired the oxidative burst, whereas the reduction in *SlCEBiP* transcripts had little to no effect (Fig. 4a). To confirm these results, we tested the induction of the three chitin responsive genes *SlBAP2*, *SlERF5*, and *SlCAL-like* in mock- and GN6-treated leaf discs of TRV-treated plants[36,37]. Preliminary results show that similar to the oxidative burst, chitin-induced gene expression is impaired in *SlLYK4*-silenced plants compared to the TRV:*GUS* controls (Fig. 4b-d). These findings suggest that *SlLYK4*, but not *SlCEBiP*, plays a role in tomato chitin recognition.

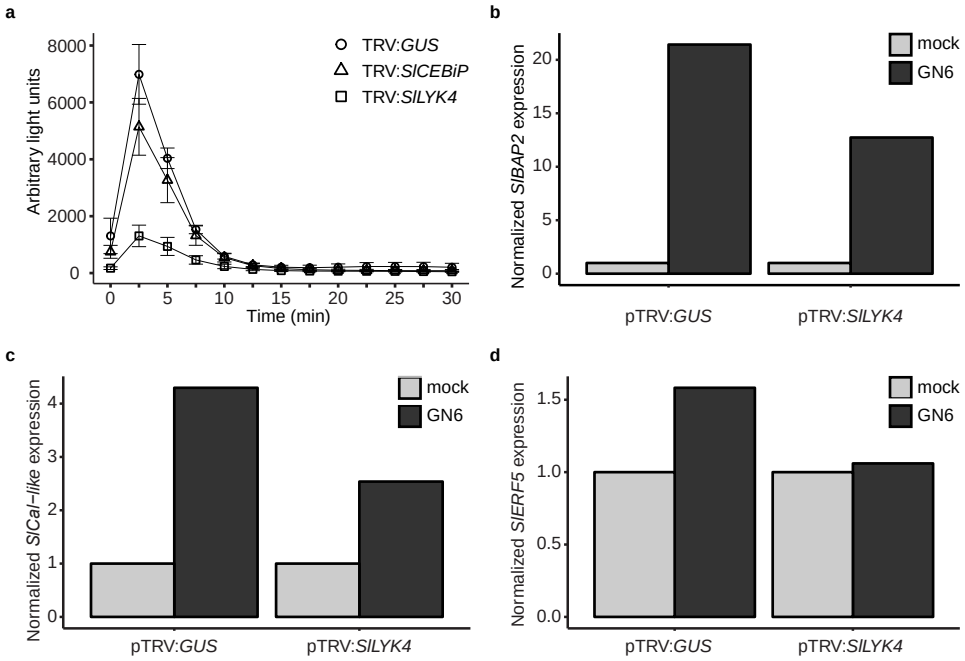

FIGURE 4 | Silencing of *SlLYK4* results in a reduction of chitin responsiveness in tomato. a, Generation of ROS was measured in leaf discs of ten TRV-treated tomato plants for 30 min following elicitation with 10 µM chitohexamer (GN6) using a luminol-based chemiluminescencec assay. Plotted are chemiluminescence averages of *n*=80 leaf discs ± S.E. normalized to water treatments. The graph is representative of three independent experiments. **b-d**, Eight leaf disks of three TRV-treated tomato plants were treated either with 10 µM GN6 or water as negative control. One hour after treatment the samples were pooled for each treatment. GN6-triggered changes in the expression of *SlBAP2* (**b**), *SlERF5* (**c**) and *SlCAL-like* (**d**) (encoding homologs of chitin-responsive Arabidopsis genes[36]) were determined by qRT-PCR. The bars display normalized transcript levels of chitin-responsive genes relative to the constitutively expressed tomato tubulin gene.

Discussion

LysM receptor proteins mediate recognition of a broad range of microbe-derived structural patterns, including bacterial peptidoglycan, fungal chitin and derivatives thereof[2,4,38]. The perception of such patterns triggers plant responses that either lead to the establishment of a mutually beneficial plant-microbe interaction or to the activation of plant immunity that restricts microbial colonization[2]. Here we identified two chitin-binding LysM receptors from tomato and investigated their role in the activation of chitin-triggered immune responses.

Affinity purification with chitin beads resulted in the recovery of the two receptor candidates SlCEBiP and SlLYK4 (Table 1). Surprisingly, both candidates eluted following treatment with GN6 and with chitosan. In contrast to chitin, chitosan oligomers have previously been reported to be only weak immunity elicitors and weak competitors of radiolabeled chitopentaose in tomato[39]. However, the role of chitosan as an inducer of immune responses remains controversial, as it has been reported to have strong effects on other plant species[40,41]. Due to the structural relatedness of chitin and chitosan, we cannot exclude that tomato chitin receptors have some affinity for chitosan, which resulted in the (partial) elution of SlLYK4 and SlCEBiP from chitin beads.

Despite its ability to bind chitin, silencing of *SlCEBiP* did not result in a reduction of chitin-triggered generation of ROS (Fig. 4a). This suggests that SlCEBiP is not part of the chitin receptor complex that activates the canonical chitin signal transduction pathway in tomato. Similar to SlCEBiP, its closest homolog AtLYM2 was identified based on its chitin-binding activity[32,42]. However, *lym2* mutants were not affected in AtCERK1-mediated chitin responses suggesting that AtLYM2 functions in a CERK1-independent pathway[19]. Indeed, AtLYM2 regulates chitin-triggered plasmodesmatal fluxes, which are required for resistance to the fungal pathogen *Botrytis cinerea*[19]. Thus, it is possible that also SlCEBiP has a more specialized role in chitin perception.

In contrast to SlCEBiP, SlLYK4 is required for the chitin-triggered generation of ROS (Fig. 4a). Additionally, silencing of *SlLYK4* impaired the induction of chitin-responsive genes (Fig. 4b-d). However, this experiment was carried out only once and requires repetition. Phylogenetic analysis showed that SlLYK4 is closely related to the Arabidopsis receptor kinases AtLYK4 and AtLYK5 (Fig. 1)[30]. Like its Arabidopsis orthologues, SlLYK4 belongs to the group of LYR receptor kinases that lack a glycine-rich loop and display a degenerate HRD motif in their catalytic loop, rendering their kinase domains inactive[11,12,30]. Since its kinase lacks the important structural features for functionality, it is likely that SlLYK4 associates with a co-receptor upon ligand perception which is reminiscent of other LYR receptors[2]. However, we did not identify a LysM receptor protein with an active kinase domain following affinity purification with chitin beads suggesting that this co-receptor may not directly be involved in ligand binding. In rice, chitin binding is mediated by

OsCEBiP[17,43], whereas OsCERK1 is recruited into the receptor complex following sandwich-like dimerization of two OsCEBiP molecules around the chitin ligand[16]. When challenged with fungal pathogens, tomato plants silenced for the closest CERK1 homolog SlLYK1 did not display marked differences in resistance compared to control plants (A. Kombrink, *personal communication*). This indicates that SlLYK1 is not part of the chitin receptor complex. However, we cannot rule out that gene silencing was insufficient to result in reduced resistance to fungal pathogens. Thus, the identification of additional components of the chitin receptor complex in tomato may require transgenic knockout tomato lines that can be assessed for their ability to mount immunity in response to fungal invasion.

Methods

Receptor candidate purification

Microsomal fractions were prepared from tomato (*Solanum lycopersicum*) cv. Heinz 1706 leaf tissue and used for chitin or chitosan affinity enrichment as described previously[32]. Proteins from unbound, eluent and bead fractions were separated by SDS-PAGE. Gel slices containing proteins of 50-100 kDa were utilized for tryptic digestion and purification for LC-MS/MS.

Candidate identification by liquid chromatography-mass spectrometry

LC-MS analysis was performed with an Orbitrap Velos Pro™ Hybrid Ion Trap-Orbitrap mass spectrometer. 1-5 µl of peptide solutions were loaded and washed on an Acclaim® PepMAP 100 pre-column (#164564, 100 µm x 2 cm, C18, 3µm, 100 Å, Thermo Fisher Scientific) with 100% loading solvent A (98% H_2O, 2% acetonitrile, 0.07% TFA) at a flow rate of 25 µl/min for 6 min. Peptides were separated by reverse phase chromatography on an Acclaim® PepMAP RSLC column (#164540, 75 µm x 50 cm, C18, 3 µm, 100 Å, Thermo Fisher Scientific) with a gradient from 98% solvent A (H_2O, 0.1% formic acid) and 2% solvent B (80% acetonitrile, 20% H_2O, 0.1% formic acid) to 42% solvent B for 95 min and to 65% solvent B for the following 26 min at a flow rate of 300 nl/min. Peptides eluting from the chromatographic column were on-line ionized by nanoelectrospray at 2.4 kV with the Nanospray Flex Ion Source (Thermo Fisher Scientific). Full scans of the ionized peptides were recorded within the Orbitrap FT analyzer of the mass spectrometer within a mass range of 300-1850 m/z at a resolution of 30.000. Collision-induced dissociation fragmentation of data-dependent top-fifteen peptides was performed with the LTQ Velos Pro linear ion trap. Data acquisition and programming were carried out with the XCalibur *2.2* software (Thermo Fisher Scientific). A UniProt-derived *Solanum lycopersicum*-specific database (http://www.uniprot.org, Proteome ID UP000004994, 33952 entries) was used

for database searches with SequestHT and Mascot search engines. Proteins were identified with the Proteome Discoverer™ 1.4 software. The digestion mode was set to trypsin and the maximum of missed cleavage sites to two. Carbamidomethylation of cysteins was set as fixed modification, oxidation of methionines, and biotinylation of lysines were set as variable modifications (if required). The mass tolerance was 10 ppm for precursor ions and 0.6 Da for fragment ions. The decoy mode was revert with a false discovery rate of 0.01.

Phylogeny of LysM receptors and protein sequence analyses

Selected sequences of LysM domain-containing protein sequences were retrieved from the solgenomics network (http://solgenomics.net) for tomato (ITAG3.0), from TAIR (http://arabidopsis.org) for Arabidopsis, from Phytozome 12 (http://phytozome.jgi.doe.gov) for rice (v7) and *Medicago truncatula* (Mt4.0v1), and UniProt (http://uniprot.org) for *Lotus japonicus*. The sequences were loaded into the "one click" phylogeny.fr server for phylogenetic analysis, ignoring alignment curation by Gblocks[44].

The sequences of SlLYK4 and SlCEBiP were further analysed with SignalP 4.1 and TargetP 1.1 (http://www.cbs.dtu.dk/services/) to predict the presence of signal peptides and extracellular localization of both candidates. The localization of the LysM domains in the extracellular region of SlLYK4 was adopted from a previous study[30], whereas the LysM domains of SlCEBiP were predicted with InterPro (http://www.ebi.ac.uk/interpro/). The GPI modification site in SlCEBiP was predicted with the big-PI plant server (http://mendel.imp.ac.at/gpi/plant_server.html).

Candidate expression analysis

Tissue-specific RNAseq data were retrieved from the tomato functional genomics database (http://ted.bti.cornell.edu) for the tomato cultivar Heinz, and from the GEO database (https://www.ncbi.nlm.nih.gov.geo.query/acc.cgi?acc=GSE33507) for the tomato cultivar Moneymaker (MM)[45].

To confirm the expression of the tomato chitin receptor candidate genes, shoot tissue was collected from nine tomato *cv*. Moneymaker plants infected with *Verticillium dahliae* strain JR2 at 4, 8 and 12 days post inoculation (dpi). Tissues from three plants were pooled for RNA isolation using the TRIZOL reagent (Invitrogen) according to manufacturer's instructions. Expression of *SlLYK4*, *SlCEBiP*, and tomato tubulin (*SlTUB*) was analysed by real-time PCR as described previously[36] using primer pairs shown in Supplementary Table 1. RNA was used as template to confirm the absence of contamination by genomic DNA. Expression levels in shoot tissues of *Verticillium*-infected plants were calculated relative to *SlTUB* using the $E^{-\Delta Ct}$ method[46] and normalized to mock-treated plants.

Virus-induced gene silencing of receptor candidates

Selected sequences of *SlLYK4* and *SlCEBiP* were amplified from tomato cv. Moneymaker cDNA and cloned into the pTRV2 vector[47] using the Gateway® technology (for primer sequences see Supplementary Table 1). Constructs were confirmed by sequencing (Supplementary Fig. 4) and transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Control TRV:*GUS* and TRV:*PDS* vectors had been previously generated and transformed into *A. tumefaciens*[48]. To silence *SlLYK4* and *SlCEBiP*, cotyledons of 10 day-old tomato cv. Moneymaker seedlings were infiltrated with 1:1 mixtures of pTRV1 and pTRV2 constructs[49]. Photobleaching was observed 10-14 days after agroinfiltration of TRV:*PDS*. At 21 dpi leaf tissue was harvested for physiological assays.

Oxidative burst and chitin-responsive gene expression assays

Oxidative burst measurements were performed on eight leaf discs from TRV-treated tomato plants as previously described[50]. Water was replaced with 100 µL of a solution containing 100 µM L-012 substrate and 20 µg/mL horseradish peroxidase. Luminescence was measured following treatment with 10 µM chitohexaose (GN6; Megazyme) or water over 30 min using a CLARIOstar® microplate reader (BMG LABTECH). Leaf discs were kept in elicitor solutions for additional 30 min and then harvested and snap-frozen in liquid N_2 for RNA extraction as described above. Chitin-responsive gene expression was tested as described previously[36].

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Author contributions

H.R. and B.P.H.J.T. conceived the project. H.R., E.K.P. and B.P.H.J.T. designed the experiments. H.R., G.C.M.B., M.E., and O.V. performed the experiments and analyzed the data. H.R. and B.H.P.J.T. wrote the manuscript.

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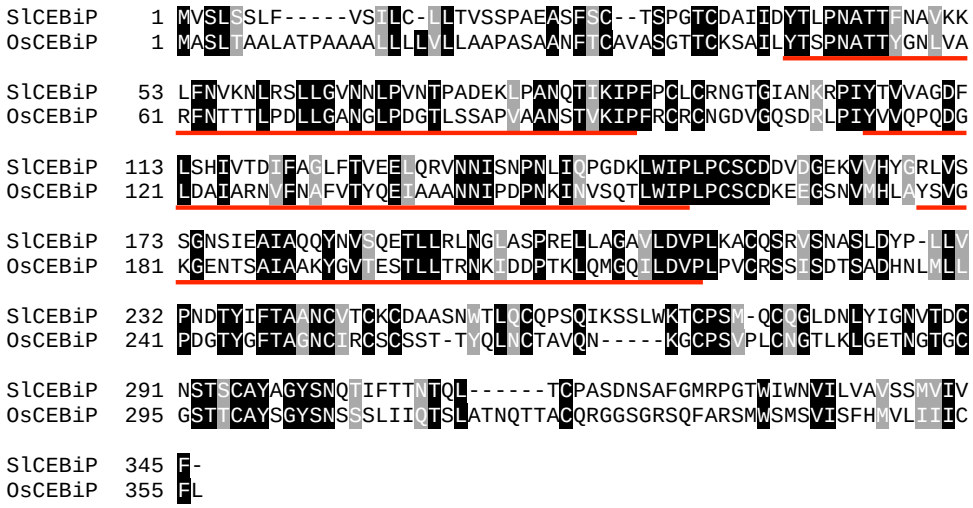
Supplementary data

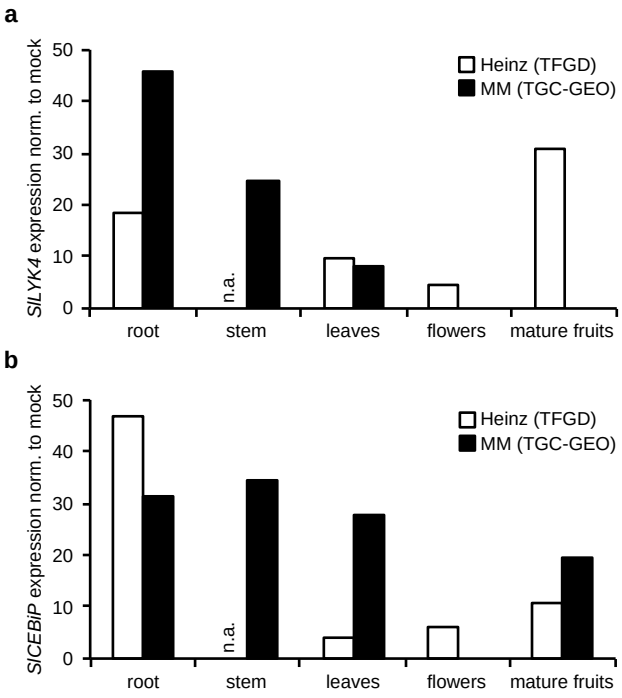
>Solyc02g089900.1.1 | mature SlLYK4
QQPYFGTGTNDCSSQDTSTSAFGYLCNGVNRTCQSYLTFRSQPPFNTVSSISSLLGANPSQLSQLNSV
SQNATFNTNQMVLVPVTCSCSGQFYQSNASYVIRRDDSFLNIAMNTLQGLSTCQAINAENSEQANNLV
VGSRINVPLRCACPTQNQTNNGTNYLLTYLIASGEFVSFISDKFGVDFRATLAANSIPEDAPTVFPNT
TLLVPLSTPPLSSQVAGPSPPPPPATTPTPPAVPVSESSSNKTWIYVVAGVVGGLVALCILGVVVFFL
FFRKKEKKADPQFVSESFEAVEKPSNKKVEEESEEFLESLSSIAQSVKVYKFEEVKAATENFSPTCLI
KGSVYRGTINGDFAAIKKMSGDVSKEINLLSKINHFNLISLSGICFHDGHWYLVYEYAANGPLSDWIC
HHNGEQKSLSWAQRVQISFDVATGLNYLHSYTSPPHVHKDLNGDNILLDGDLRAKIANFGLARSADGQ
EGEFALTRHIVGTQGYMAPEYLENGLVSPKLDVYALGVLLLEILTGKEVSALYEGSNTNLAELLIPVL
NDDNAKESLSNFVDPSLQGKYPVELAFAMVRLIDNCLMKDPSHRPNTDEIVQSVSRIMTATHSWETSF
STSVSPHRLP

>Solyc01g112080.2.1 | mature SlCEBiP
SFSCTSPGTCDAIIDYTLPNATTFNAVKKLFNVKNLRSLLGVNNLPVNTPADEKLPANQTIKIPFPCL
CRNGTGIANKRPIYTVVAGDFLSHIVTDIFAGLFTVEELQRVNNISNPNLIQPGDKLWIPLPCSCDDV
DGEKVVHYGRLVSSGNSIEAIAQQYNVSQETLLRLNGLASPRELLAGAVLDVPLKACQSRVSNASLDY
PLLVPNDTYIFTAANCVTCKCDAASNWTLQCQPSQIKSSLWKTCPSMQCQGLDNLYIGNVTDCNSTSC
AYAGYSNQTIFTTNTQLTCPASDNSAFGMRPGTWIWNVILVAVSSMVIVF

SUPPLEMENTARY FIGURE 1 | Coverage map of SlLYK4 and SlCEBiP peptides identified by mass spectrometry. Peptides identified by LC-MS and mapping to SlLYK4 or SlCEBiP protein sequences are highlighted in grey. The first amino acid residue of the transmembrane domain of SlLYK4 is underlined (W248).



SUPPLEMENTARY FIGURE 2 | Pairwise alignment of rice and tomato CEBiP protein sequences. Full-length protein sequences of SlCEBiP and OsCEBiP were aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). LysM domains as annotated for OsCEBiP[16,33] are underlined in red. Sequence conservation between the two receptors suggests that SlCEBiP contains three LysM domains.



SUPPLEMENTARY FIGURE 3 | Expression of *SlLYK4* and *SlCEBiP* in different tomato tissues. Tissue-specific expression patterns of *SlLYK4* (**a**) and *SlCEBiP* (**b**) determined by RNAseq in the tomato cultivars Heinz 1706 and Moneymaker (MM) extracted from the tomato functional genomics database (TFGD) and the tomato genome consortium GEO (TGC-GEO) databases, respectively.

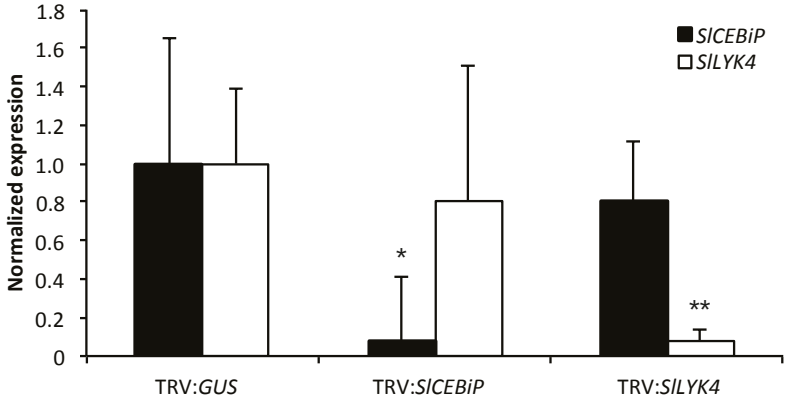
a

```
SlCEBiP_VIGS    1 --------------------------------------------------CCATTTACA
SlCEBiP_temp  481 TCCCCTTTCCTTGTCTCTGTAGAAACGGTACCGGAATAGCCAACAAACGCCCCATTTACA

SlCEBiP_VIGS   10 CCGTCGTCGCCGGCGACTTCCTATCGCACATAGTTACCGACATCTTCGCCGGTTTGTTCA
SlCEBiP_temp  541 CCGTCGTCGCCGGCGACTTCCTATCGCACATAGTTACCGACATCTTCGCCGGTTTGTTCA

SlCEBiP_VIGS   70 CTGTTGAGGAACTTCAAAGGGTTAACAATATATCTAACCCCAATTTGATACAACCCGGGG
SlCEBiP_temp  601 CTGTTGAGGAACTTCAAAGGGTTAACAATATATCTAACCCCAATTTGATACAACCCGGGG

SlCEBiP_VIGS  130 ATAAATTGTGGATCCCACTTCCTTGCAGCTGCGACGACGTTGACGGTGAAAAAGTTGTTC
SlCEBiP_temp  661 ATAAATTGTGGATCCCACTTCCTTGCAGCTGCGACGACGTTGACGGTGAAAAAGTTGTTC

SlCEBiP_VIGS  190 ATTATGGTCGATTGGTGAGCAGTGGCAACAGTATTGAGGCTATTGCTCAGCAGTACAATG
SlCEBiP_temp  721 ATTATGGTCGATTGGTGAGCAGTGGCAACAGTATTGAGGCTATTGCTCAGCAGTACAATG

SlCEBiP_VIGS  250 TTTCCCAGGAAACCCTCTTGAGGTTGAATGGTTTAGCAAGTCCCAGAGAACTTTTAGCTG
SlCEBiP_temp  781 TTTCCCAGGAAACCCTCTTGAGGTTGAATGGTTTAGCAAGTCCCAGAGAACTTTTAGCTG

SlCEBiP_VIGS  310 GCGCAGTTCTTGACGTTC----------------------------------------
SlCEBiP_temp  841 GCGCAGTTCTTGACGTTCCCCTTAAAGCTTGCCAATCAAGGGTGAGCAATGCCTCGCTGG
```

b

```
SlLYK4_VIGS     1 ------------------------GTTTTTACCATCATTCTTGCTTATTCCTCTGTTTCA
SlLYK4_temp     1 ATGAATTATTCTCATCTCATCTTTGTTTTTACCATCATTCTTGCTTATTCCTCTGTTTCA

SlLYK4_VIGS    37 ATTCTTGCACAACAGCCTTATTTTGGAACTGGAACAAATGACTGCAGCAGCCAAGATACC
SlLYK4_temp    61 ATTCTTGCACAACAGCCTTATTTTGGAACTGGAACAAATGACTGCAGCAGCCAAGATACC

SlLYK4_VIGS    97 TCCACTTCTGCTTTTGGGTATTTATGCAATGGCGTTAACCGTACTTGCCAATCTTATTTG
SlLYK4_temp   121 TCCACTTCTGCTTTTGGGTATTTATGCAATGGCGTTAACCGTACTTGCCAATCTTATTTG

SlLYK4_VIGS   157 ACCTTCAGATCTCAACCCCCTTTCAATACTGTGTCCTCAATCTCTTCTTTACTCGGTGCT
SlLYK4_temp   181 ACCTTCAGATCTCAACCCCCTTTCAATACTGTGTCCTCAATCTCTTCTTTACTCGGTGCT

SlLYK4_VIGS   217 AATCCTTCACAGCTCTCTCAGCTCAATTCTGTTTCTCAAAATGCTACCTTTAACACCAAT
SlLYK4_temp   241 AATCCTTCACAGCTCTCTCAGCTCAATTCTGTTTCTCAAAATGCTACCTTTAACACCAAT
```

SUPPLEMENTARY FIGURE 4 | Alignment of target sequences selected for virus-induced gene silencing with gene templates. Prior to transformation of *A. tumefaciens* pTRV2 vectors carrying gene fragments of *SlCEBiP* (**a**) and *SlLYK4* (**b**) were verified by sequencing.



SUPPLEMENTARY FIGURE 5 | Efficiency and specificity of *SlLYK4* and *SlCEBiP* silencing in tomato. Silencing efficiency of *SlCEBiP* and *SlLYK4* was determined by qRT-PCR in tomato leaves three weeks following tobacco rattle virus (TRV) treatment using gene-specific primers. Primers targeting the tomato tubulin gene were used for sample calibration. Relative transcript levels were set to 1 for TRV:*GUS*-treated samples for each gene. Bars represent expression levels in $n>3$ plants ± S.D.. Statistically significant differences in expression compared to TRV:*GUS* plants were determined with a Student's *t* test (*p<0.5; **p<0.01).

SUPPLEMENTARY TABLE 1 | Primers used in this study.

Name	Sequence (5' → 3')	Application
SlLYK4_qPCR_F	TCAACGCGGAGAACAGTGAA	qRT-PCR
SlLYK4_qPCR_R	GCCCTAAAATCCACCCCAAA	qRT-PCR
SlCEBiP_qPCR_F	CTTGCCAATCAAGGGTGAGC	qRT-PCR
SlCEBiP _qPCR_R	ATCTGGGATGGTTGGCATTG	qRT-PCR
SlTUB_F	AACCTCCATTCAGGAGATGTTT	qRT-PCR
SlTUB_R	TCTGCTGTAGCATCCTGGTATT	qRT-PCR
SlLYK4_VIGS_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAATTATTCTCATCTC	VIGS
SlLYK4_VIGS_R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCATTATTAATTGGTGTTAAAGGTAGC	VIGS
SlCEBiP_VIGS_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATTTACACCGTCGTCGC	VIGS
SlCEBiP _VIGS_R	GGGGACCACTTTGTACAAGAAAGCTGGGTGAACGTCAAGAACTGCGCC	VIGS

Chapter 5

Neofunctionalisation after horizontal gene transfer: birth of a fungal effector that evolved towards immune suppression

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[#]These authors contributed equally

Abstract

Microbes utilize secreted molecules to facilitate niche establishment, including microbial pathogens that colonize plant hosts[1]. We previously identified the secreted effector protein Ave1 of the vascular wilt fungus *Verticillium dahliae* that acts as a virulence factor during plant colonization[2]. Ave1 homologs are ubiquitous in plants and occur only in few plant pathogens that acquired them via horizontal gene transfer (HGT)[2]. Intriguingly, all homologs carry a conserved plant natriuretic peptide (PNP) sequence. Here we show that, as previously demonstrated for some of its homologs, also *V. dahliae* Ave1 displays PNP activity. However, the contribution of Ave1 to virulence is not dependent on its PNP activity. Further analysis revealed that Ave1 interacts with plant endochitinases, which are important executors of plant defense[3]. Intriguingly, in contrast to its homologs, Ave1 inhibits endochitinases to interfere with host immunity. Thus, Ave1 functionally diverged from its microbial and plant homologs upon HGT from plants to evolve a novel function in immune suppression.

Introduction

Verticillium dahliae is a soil-inhabiting fungal pathogen that causes vascular wilt in over 200 dicotyledonous plants species, including many crops[4,5]. Like other microbes, *V. dahliae* secretes effector molecules to facilitate niche establishment[1,6]. Using comparative population genomics, we identified the *V. dahliae* effector gene *Ave1* (hereafter referred to as *VdAve1*) that is crucial for fungal aggressiveness during infection (virulence) on tomato (*Solanum lycopersicum*), on *Arabidopsis thaliana*[2], and on other host plants (B.P.H.J. Thomma, unpublished data). Similarity searches revealed that VdAve1 has numerous homologs in plants, but also in the phytopathogenic fungi *Fusarium oxysporum*, *Colletotrichum higginsianum*, and *Cercospora beticola*, in the saprotroph *Verticillium nubilum* as well as in the bacterial pathogen *Xanthomonas citri* subsp. *citri*[2,7]. Since the distribution of VdAve1 homologs does not follow the phylogeny of the species in which they occur, it has been proposed that *V. dahliae* acquired *VdAve1* from plants by horizontal gene transfer (HGT)[2]. This hypothesis is further supported by its localization in a highly dynamic lineage-specific region of the *V. dahliae* genome[8-10].

Results and discussion

Most plant homologs of VdAve1 are annotated as plant natriuretic peptides (PNPs)[11,12]; functional analogues of vertebrate atrial natriuretic peptides (ANPs) that contribute to maintenance of osmotic and cardiovascular homeostasis[13]. Similarly, PNPs are systemically mobile molecules that are released under biotic and abiotic stress conditions and have been implicated in ion and water homeostasis[12,14]. Interestingly, the VdAve1 homolog XacPNP that is produced by the plant pathogenic bacterium *X. citri* subsp. *citri* is a horizontally acquired virulence factor that promotes bacterial proliferation through its PNP activity[15,16]. An alignment of VdAve1 with homologs of *V. nubilum* (VnAve1), *C. beticola* (CbAve1), *F. oxysporum* (FoAve1), *C. higginsianum* (ChAve1), XacPNP, tomato (SlAve1), and *A. thaliana* (AtPNP-A) shows that the smallest peptide with PNP function lies within the most conserved region (grey box, Fig 1a;[15]). To test whether VdAve1 has PNP activity, its ability to promote stomatal opening was assayed in tomato (Fig. 1b). Treatment of leaf epidermis with VdAve1 resulted in significantly enhanced stomatal opening as similarly observed upon treatment with XacPNP and the synthetic auxin analogue naphthalene acetic acid (NAA) (Fig 1b). Consistent with previous reports demonstrating that PNP-induced responses are dependent on cyclic guanosine monophosphate (cGMP) signalling[17], aperture changes caused by VdAve1 were partially repressed by the guanylate cyclase inhibitor methylene blue. Collectively, these findings demonstrate that VdAve1 displays PNP activity.

To test whether the PNP activity accounts for the virulence activity of VdAve1, we complemented a *VdAve1* deletion mutant (Δ*VdAve1*)[2] with *VnAve1*, *ChAve1*, *FoAve1*, *CbAve1*, *XacPNP*, or *SlAve1* under control of the *VdAve1* promoter (Supplementary Fig. 1a), and confirmed transgene expression by qRT-PCR (Supplementary Fig. 1b). For each construct, we selected two transformants of which at least one reached an expression value similar to wild type *VdAve1* for inoculation assays on tomato. Surprisingly, none of the VdAve1 homologs was able to restore the virulence of the *VdAve1* deletion mutant to wild type, as determined by assessment of symptom display of inoculated plants (Fig. 2a) and fungal biomass by qRT-PCR (Fig. 2b). Since the PNP domain is highly conserved in all homologs tested here and is functional in VdAve1 as well as XacPNP, these results suggest that the PNP activity of VdAve1 is not responsible for its role in virulence, and that this effector has acquired an additional function to facilitate host colonization.

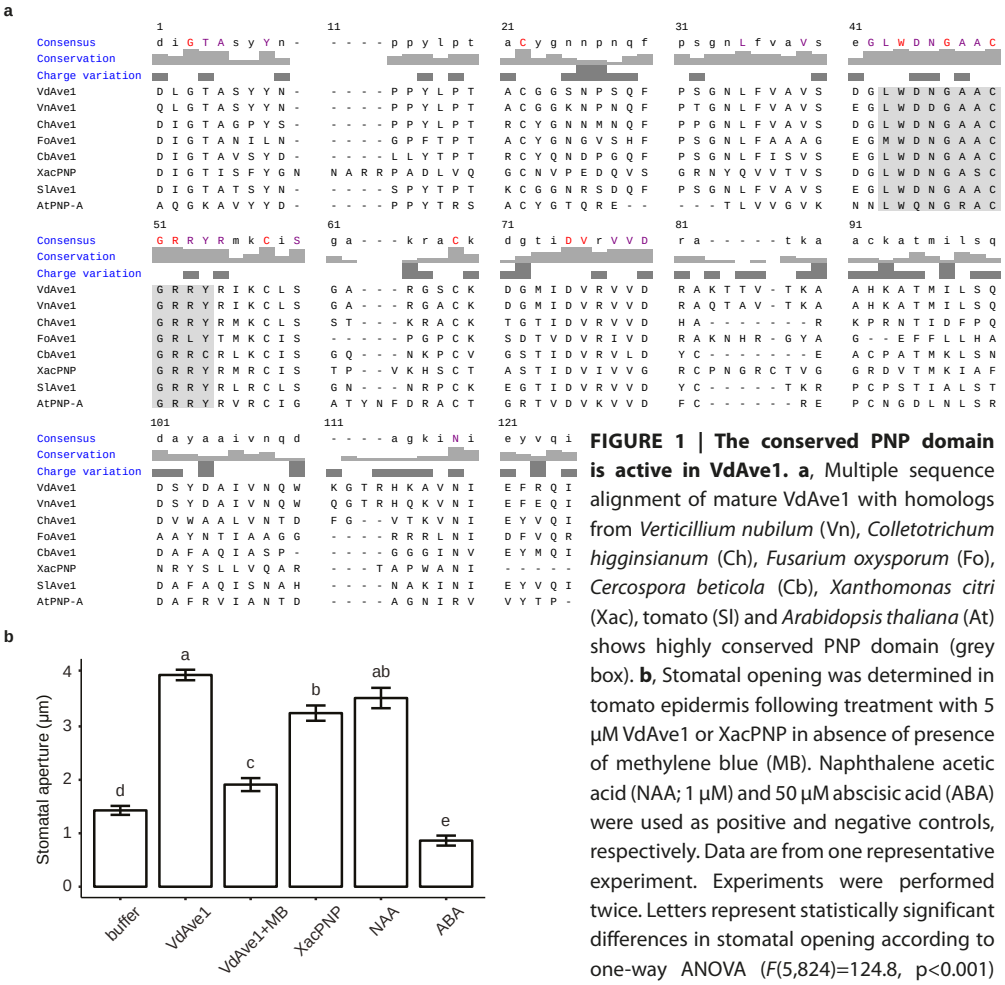

FIGURE 1 | The conserved PNP domain is active in VdAve1. a, Multiple sequence alignment of mature VdAve1 with homologs from *Verticillium nubilum* (Vn), *Colletotrichum higginsianum* (Ch), *Fusarium oxysporum* (Fo), *Cercospora beticola* (Cb), *Xanthomonas citri* (Xac), tomato (Sl) and *Arabidopsis thaliana* (At) shows highly conserved PNP domain (grey box). **b**, Stomatal opening was determined in tomato epidermis following treatment with 5 µM VdAve1 or XacPNP in absence of presence of methylene blue (MB). Naphthalene acetic acid (NAA; 1 µM) and 50 µM abscisic acid (ABA) were used as positive and negative controls, respectively. Data are from one representative experiment. Experiments were performed twice. Letters represent statistically significant differences in stomatal opening according to one-way ANOVA ($F(5,824)=124.8$, $p<0.001$) and Tukey's post-hoc test. Error bars represent the mean ± SD (n>70).

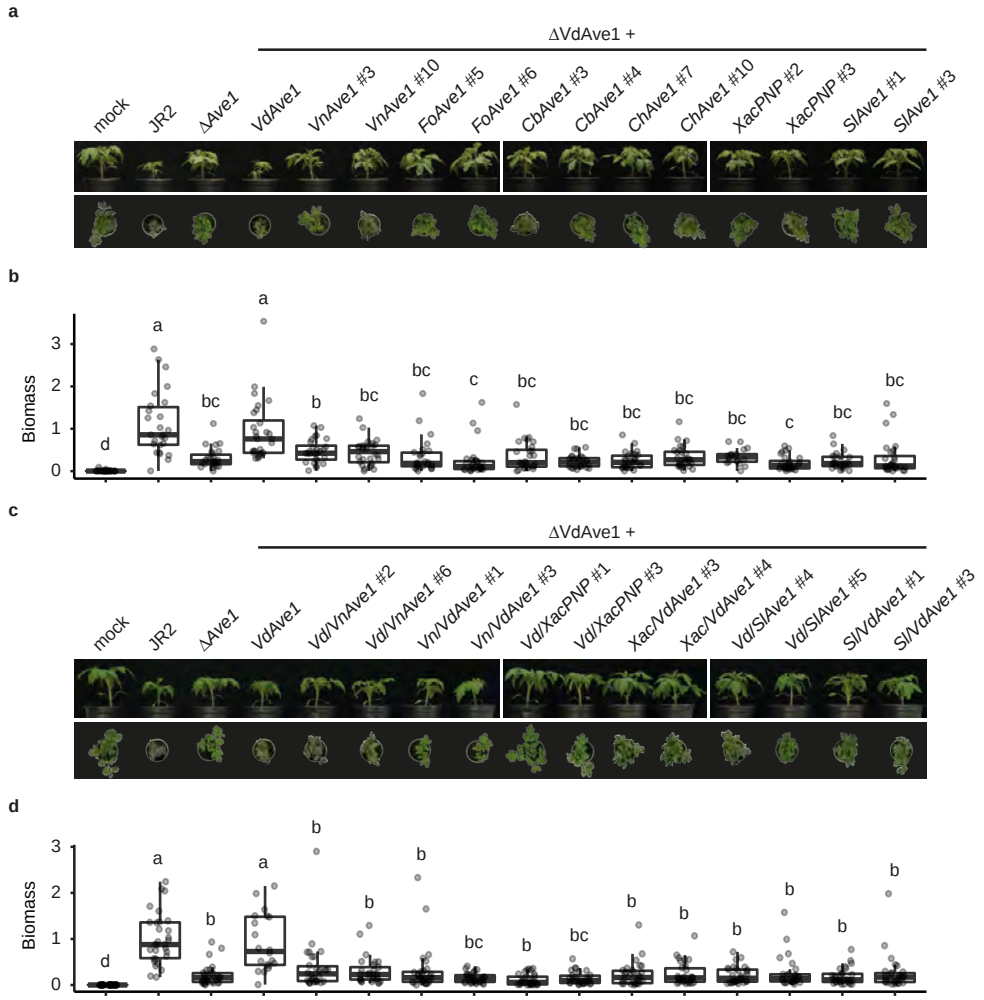

FIGURE 2 | VdAve1 has functionally diverged from plant and microbial homologs. Complementation assays in the *V. dahliae Ave1* deletion mutant with microbial and plant homologs (**a**,**b**; Supplementary Fig. 1) and chimeric mutants (**c**,**d**; Supplementary Fig. 2) of Ave1 show that full-length VdAve1 is required for *V. dahliae* virulence during tomato colonization. **a**, Representative stunting symptoms of plants inoculated with wild type *V. dahliae* (JR2), Δ*VdAve1*, Δ*VdAve1* complemented with *VdAve1*, or two independent Δ*VdAve1* strains expressing *VdAve1* homologs from *V. nubilum* (Vn), *C. higginsianum* (Ch), *C. beticola* (Cb), *F. oxysporum* (Fo), *X. citri* (Xac), and tomato (Sl). The *XacPNP* sequence encoding the mature protein was fused to the *VdAve1* signal peptide to ensure secretion. Plants inoculated with strains expressing *VdAve1* homologs display stunting symptoms similar to plants inoculated with the *VdAve1* deletion strain, suggesting that the homologs fail to reinstall virulence. **b**, Fungal biomass was quantified in stem tissue of all mock-treated and *V. dahliae*-inoculated plants by quantitative RT-PCR on genomic DNA targeting the *V. dahliae ITS* gene at 17-19 days after inoculation. Primers targeting the tomato rubisco gene were used for sample calibration. Data are from three independent experiments. Letters represent statistically significant differences in biomass according to one-way ANOVA ($F(15,399)=19.988$, $p<0.001$) and Tukey's post-hoc test. Error bars represent the mean ± SD (n>26). **c**, Similar to full-length homologs, complementation of the *VdAve1* deletion mutant with constructs expressing chimeric Vn/VdAve1, Vd/VnAve1, Xac/VdAve1, Vd/XacPNP, Sl/VdAve1 and Vd/SlAve1 did not result in the recovery of virulence. **d**, Quantification of fungal biomass as in **b**. Data are from three independent experiments. Letters represent statistically significant differences in biomass according to one-way ANOVA ($F(15,435)=67.084$, $p<0.001$) and Tukey's post-hoc test. Error bars represent the mean ± SD (n>29).

We then generated a range of constructs for the expression of domain swap mutants between VdAve1 and VnAve1, XacPNP or SlAve1 in the *VdAve1* deletion mutant to investigate which portion of VdAve1 is responsible for its virulence function (Supplementary Fig. 2a). The swapping sites were chosen such that the most conserved sequence, including the active PNP domain, originated from the VdAve1 homologs. Again, transformants were selected based on transgene expression as described above (Supplementary Fig. 2b). Intriguingly, symptom assessment and quantification of fungal biomass in infected plants showed that, similar to the full-length homologs, none of the chimeric Ave1 proteins reinstalled virulence in Δ*VdAve1* during tomato colonization (Fig. 2c,d). This finding confirms that the PNP domain is not responsible for the virulence function of VdAve1. Moreover, we hypothesize that the domain swaps disrupted the domain required for the virulence activity of VdAve1. Based on the functional dissection of the VdAve1 epitope, we have recently proposed that the surface that is formed by the N- and C-termini of VdAve1 is crucial for the recognition by the tomato immune receptor Ve1[7]. Taken together, these findings suggest that a modification of either terminus of the protein destabilizes its overall structure, affecting both VdAve1 immune recognition and its virulence function.

Considering that the PNP activity is not responsible for the virulence contribution of VdAve1, and that we cannot attribute the virulence function to a specific subdomain of the effector protein, we pursued affinity purification and identification of interacting plant proteins by mass spectrometry to reveal how VdAve1 contributes to virulence. To this end, we transiently expressed VdAve1 fused to green fluorescent protein (GFP), which was previously shown to be recognized by Ve1, in tobacco (*Nicotiana tabacum*) leaves[7]. The transiently expressed proteins were immunopurified with αGFP agarose beads and peptides generated by tryptic on-bead digestion were analyzed with mass spectrometry. While twelve proteins specifically co-purified with VdAve1, only two tobacco endochitinases (CHI1 and CHI2), with an average of 4 unique peptides in four biological replicates, were considered as *bona fide* interactors, since the remaining proteins are predicted to be localized intracellularly while VdAve1 is thought to act extracellularly (Fig. 3a; Supplementary Fig. 3; Supplementary Table 2). Endochitinases are important pathogenesis-related proteins and play a key role in plant defense against fungal microbes[3,18]. Consequently, several mechanisms evolved in fungi that enable them to evade or suppress host chitinase activity, such as proteolytic cleavage of chitinases and competition for the binding of chitinase substrates[19-21].

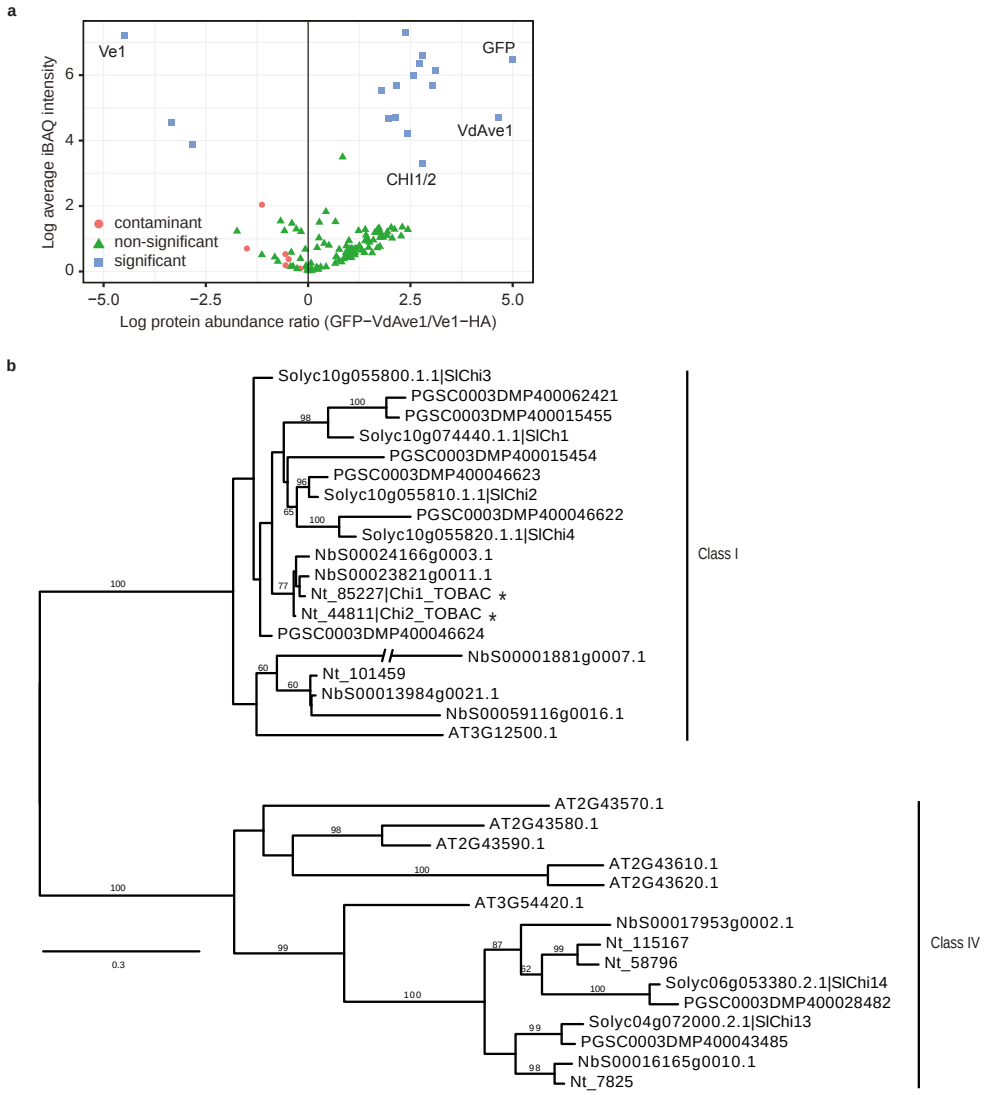

FIGURE 3 | ChtBD1 domain-carrying tomato endochitinases are putative targets of VdAve1. a, Putative targets of VdAve1 were identified by mass spectrometry following transient expression of GFP-tagged VdAve1 in tobacco leaves. Leaves of four plants were independently infiltrated with *A. tumefaciens* carrying *GFP-Ave1* or *Ve1-HA* constructs and treated as biological replicates in subsequent analyses. Immunopurification of tagged proteins was carried out on total leaf extracts 2 days after infiltration. The secreted tobacco endochitinases CHI1 and CHI2 specifically co-purified with GFP-Ave1. **b**, Phylogeny of putative secreted endochitinases of tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), *Nicotiana benthamiana*, potato (*Solanum tuberosum*), and *Arabidopsis thaliana* carrying both a chitin-binding (ChtBD1; PF00187) and a glycosyl hydrolase family 19 (GH19; PF00182) domain. Clustering shows that endochitinases group into two clades corresponding to class I and class IV chitinases. The two tobacco chitinases CHI1 and CHI2 (*) identified by mass spectrometry belong to class I, which contains four homologs from tomato. The clade of class IV chitinases only contains two putative tomato endochitinases.

The tomato genome comprises six genes encoding endochitinases (*SlChi1*, *SlChi2*, *SlChi3*, *SlChi4*, *SlChi13*, and *SlChi14*) that are closely related to tobacco CHI1 and CHI2. These are the only secreted tomato chitinases that contain both a chitin-binding type-1 (ChtBD1; PF00187) and a chitinolytic glycosyl hydrolase 19 (GH19; PF00182) domain[21]. The ability to bind chitin via their ChtBD1 domain has previously been described to enhance enzymatic efficiency of GH19 chitinases, and removal of this domain compromises their enzymatic and antifungal activity[21-23]. Phylogenetic analysis of the tobacco and tomato chitinases and predicted secreted chitin-binding chitinases from *Nicotiana benthamiana, Solanum tuberosum* (potato), and *Arabidopsis thaliana* demonstrated that they group into two clades corresponding to class I and class IV chitinases (Fig. 3b)[24]. Quantitative RT-PCR analysis showed that all six endochitinase genes are expressed in tomato stem tissue during *V. dahliae* infection (Supplementary Fig. 4). Similarly, expression of *SlChi2*, *SlChi3*, *SlChi4*, and *SlChi13* was induced during tomato infection by *F. oxysporum* that, like *V. dahliae*, colonizes the plant vascular system[21,25]. Additionally, *SlChi2*, *SlChi3*, and *SlChi4* expression was significantly upregulated in tomato leaf tissue upon inoculation with the extracellular, foliar pathogen *Cladosporium fulvum*[21,26,27]. These results indicate that the six chitin-binding tomato chitinases play an important role in defense against various fungal pathogens.

The finding that VdAve1 interacts with chitinases may suggest that *V. dahliae* utilizes this effector to inhibit host chitinases in order to protect itself from their enzymatic activity. To determine the potential inhibitory activity of VdAve1, we purified the tomato class I chitinases SlChi2 and SlChi4 upon heterologous expression from *Pichia pastoris*, and SlChi13 as representative of class IV chitinases. We first assessed the hydrolytic activity of SlChi2, SlChi4, and SlChi13 alone using a colorimetric assay. Of the three enzymes, only SlChi4 displayed endochitinolytic activity (Supplementary Fig. 5a) while its ability to hydrolyze the exochitinase-specific substrate was negligible, confirming its annotation as endochitinase. Neither SlChi2 nor SlChi13 showed measurable enzymatic activity, possibly indicating differences in substrate specificity as both chitinases were previously shown to hydrolyze insoluble chitin azure[21]. Based on these findings, SlChi4 was used in subsequent experiments. Its activity was suppressed by the chitinase inhibitor tri-N-acetylchitotriosyl moranoline (GN_3M)[28] in a concentration-dependent manner, but not by monomeric N-acetylchitotriosyl moranoline (GNM)[28] (Supplementary Fig. 5b,c), which were used as positive and negative controls, respectively. Interestingly, incubation of SlChi4 with *Escherichia coli*-produced VdAve1 resulted in significantly decreased SlChi4 activity, demonstrating that the effector indeed acts as a chitinase inhibitor (Fig. 4). Notably, 0.5 µM VdAve1 was sufficient to reduce SlChi4 activity by ~50%, which is similar to the inhibition by 100 µM GN_3M, demonstrating that VdAve1 is a considerably more potent chitinase inhibitor than GN_3M.

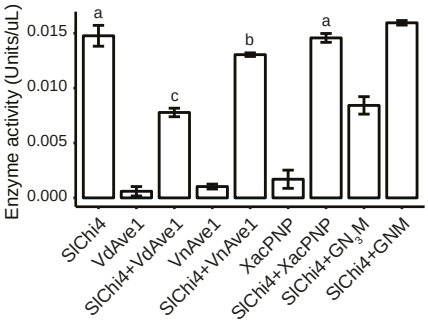

FIGURE 4 | VdAve1 inhibits tomato endochitinase activity. SlChi4 (0.1 μM) activity was quantified based on the release of *p*-nitrophenol from the endochitinase substrate 4-Nitrophenyl β-D-N,N´,N´´-triacetylchitotriose following two hours of incubation in reaction buffer (pH 5.5) at 37°C. For inhibition, SlChi4 was pre-incubated for 15 min with 0.5 μM effector proteins or 100 μM GN_3M and GNM, respectively. Data are from three independent experiments with two technical replicates. Bars represent means ± SD (*n*=3). Letters show statistically significant differences according to one-way ANOVA and Tukey's post-hoc test.

Since complementation of the *VdAve1* deletion mutant of *V. dahliae* with homologs from microbes and plants did not result in the recovery of virulence, we speculated that only VdAve1 has the capacity to inhibit endochitinase activity. To validate this hypothesis, we assessed the inhibitory activity of selected Ave1 homologs towards SlChi4. To this end, we tested the closest VdAve1 homolog, VnAve1, as well as the well-characterized homolog XacPNP, which was shown to act as a microbial virulence factor. In support of our hypothesis, only addition of VdAve1, but not of VnAve1 or XacPNP to the reaction mixture resulted in significant inhibition of SlChi4 activity (Fig. 4). Collectively, these results demonstrate that only VdAve1, in contrast to its homologs, acquired an additional functionality to suppress the hydrolytic activity of plant endochitinases.

The hydrolytic cleavage of cell wall chitin by endochitinases during plant colonization has detrimental effects on invading fungal microbes. On the one hand, it releases chitin fragments that are recognized by plant cell surface receptors, resulting in the stimulation of further immune responses[29]. On the other hand, the hydrolysis of structural chitin macromolecules affects the integrity of the fungal cell wall, which may ultimately lead to cell collapse[18]. Thus, by secreting VdAve1 during host colonization, *V. dahliae* may interfere with chitin-triggered immunity in two, not mutually exclusive, ways: (1) by inhibiting the hydrolysis of fungal hyphae, and (2) by preventing the release of immunogenic chitin oligomers. This finding explains the earlier observation that the core chitin-binding lysin motif (LysM)-containing effector proteins produced by *V. dahliae* do not interfere with chitin-triggered immunity[30]. This was surprising since all fungal plant pathogens need to address the threat posed by chitin-triggered host immunity, a role that has been attributed to the ubiquitous fungal LysM effectors in many species[20,31,32]. However, in *V. dahliae*, VdAve1 has evolved to fulfill this task.

The acquisition of effector genes by HGT from plants has been associated with emergence of pathogenicity and microbial host range expansion[33]. Moreover, phytopathogens utilize host-related molecules for immune evasion and host manipulation[15,34]. Here we show that VdAve1, in contrast to its homologs, inhibits tomato endochitinases during infection, representing a novel strategy for phytopathogens to deal with host-derived cell

wall hydrolytic enzymes. The finding of yet another strategy to undermine chitin-triggered immunity underpins its importance in host defense against fungal pathogens. Thus, we reveal a case of neofunctionalisation of a horizontally acquired effector gene toward a function in host immune suppression.

Methods

Sequence alignment of Ave1 proteins

Protein alignment was performed using MAFFT (Version 7.271)[35].

Production and purification of recombinant effector proteins

The sequences encoding mature VdAve1, VnAve1, and XacPNP were cloned into the pET-15b expression vector for N-terminal His_6 tagging (Novagen, Madison, WI, USA) (for primer sequences see Supplementary Table 1). Heterologous proteins were produced and purified from inclusion bodies under denaturing conditions using His60 Ni^{2+} Superflow Resin (Clontech, Mountain View, CA, USA). Purified proteins were stored in 0.25 M ammonium sulphate with 0.1 M BisTris, pH 5.5. Final concentrations were determined using the BioRad Protein Assay (BioRad, Veenendaal, The Netherlands). For details see Supplementary Methods.

Stomatal opening assay

Stomatal aperture was tested as described previously[15] using tomato leaf tissue. Significant differences between treatments were determined using one-way ANOVA followed by Tukey's post-hoc test in R (Version 3.3.2).

Generation of *V. dahliae* complementation strains

Genes encoding VdAve1 homologs and chimeric Ave1 proteins (Supplementary Fig. 2) were synthesized (Eurofins, Ebersberg, Germany) and cloned into the pFBT005 vector under the control of the *VdAve1* promoter (for details see Supplementary information). For expression of full-length *XacPNP* and *Xac/VdAve1*, the native signal peptide of *XacPNP* was replaced with that of *VdAve1* (see Supplementary Fig. 1a, 2a). Transformation of *V. dahliae* Δ*Ave1*[2] with *A. tumefaciens* Agl1 carrying the different constructs was performed as described previously[7,36,37]. Transgene expression was determined for individual colonies, grown in liquid ½ Murashige & Skoog medium supplemented with 3% sucrose and 1 mM MES, pH 5.7 at 22°C and 120 rpm, by qRT-PCR using primers targeting specific transgenes and *VdGAPDH* for sample calibration (Supplementary Table 1). Quantitative RT-PCR conditions and analyses of results were described previously[2].

Disease assays

Tomato inoculations were carried out as described previously[36]. Disease development was monitored up to 19 days after inoculation. *V. dahliae* biomass was quantified as described in the Supplementary Methods. Significant differences in biomass between tomato plants inoculated with *V. dahliae* wild type and mutant strains was determined by one-way ANOVA, followed by Tukey's post-hoc analysis in R (Version 3.3.2).

VdAve1 target identification by immunopurification and mass spectrometry.

Immunopurifications were carried out as described previously[38] with minor modifications (for details see Supplementary Methods). Peptides were analyzed by mass spectrometry (see Supplementary Methods). For protein identification, the publicly available tobacco (*N. tabacum*) database from Uniprot (2013) and an in-house *V. dahliae* (PeterEsse_JR2_Genome_2013) database were queried. Results were analyzed with MaxQuant (Version 1.3.0.5.) in label-free mode[39]. Result filtering and statistical analyses were carried out as described previously[40]. Tobacco CHI1 and CHI2 domain identities were confirmed using InterPro (Version 62.0; http://www.ebi.ac.uk/interpro/).

Phylogenetic analysis of plant chitinases

To discern putative chitinases closely related to the two tobacco chitinases identified by mass spectrometry, we queried the predicted proteomes of the Solanaceous plants tomato (Version 2.4), *Solanum tuberosum* (potato; Version 3.4), *Nicotiana benthamiana* (Version 0.4.4), tobacco (all from solgenomics.net), as well as *A. thaliana* (thale cress; TAIR Version 10) for proteins containing chitin-binding (PF00187) and glycoside hydrolase family 19 (PF00182) domains using HMMer (Version 3.1b)[41] and a local Pfam database (Version 27)[42]. Proteins without predicted N-terminal secretion signal and truncated proteins were removed. Remaining protein sequences were aligned using MAFFT (Version 7.271)[35]. Maximum likelihood phylogeny was inferred using RaXML (Version 8.2.4; WAG model of amino acid change and GAMMA model of rate heterogeneity)[43], and its robustness was assessed using 1,000 bootstrap replicates.

Production of recombinant tomato chitinases

Pichia pastoris GS115 expressing recombinant tomato chitinases SlChi2, SlChi4 or SlChi13[21] were grown in a New Brunswick Bioflo 3000 fermenter for protein production and purification as described previously[21,44] with minor modifications in the purification process (for details see Supplementary Methods). Purified chitinases were stored in 0.2 M NaCl and concentrations were measured as described above.

Colorimetric chitinase activity assay

Endo- and exochitinase activities of purified chitinases SlChi2, SlChi4, SlChi13 were tested using 0.2 mg/mL or 0.5 mg/mL 4-Nitrophenyl β-D-N,N´,N´´-triacetylchitotriose and N-Nitrophenyl N-acetyl-β-D-glucosaminide substrates, respectively, from the Chitinase Assay Kit (CS0980, Sigma, St. Louis, MO, USA) according to manufacturer's instructions. Effector proteins or moranoline-conjugated chitin oligomers[28] were pre-incubated with SlChi4 at indicated concentrations for 15 min prior to addition to substrates. The release of *p*-nitrophenol (4-nitrophenol) was measured after 2 hours of incubation in 0.25 M ammonium sulfate, 0.1 M BisTris, pH 5.5 at 37°C in a CLARIOstar® plate reader (BMG LABTECH, Ortenberg, Germany) at 405 nm. Absorbance values were exported from the MARS data analysis software (BMG LABTECH, Ortenberg, Germany). Enzyme activities were calculated as described in the kit's technical bulletin. Statistical differences between treatments were determined by one-way ANOVA followed by Tukey's post-hoc analysis in R (Version 3.3.2).

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Author contributions

H.R. and B.P.H.J.T. conceived the project. H.R., J.R.M. and B.P.H.J.T. designed the experiments. H.R., J.C.B., N.C.S., C.G., J.O., S.B., and M.F.S. carried out the experiments and analyzed the data. M.O. provided crucial experimental material. H.R. and B.P.H.J.T. wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary methods

Plant materials

Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) and tomato plants were grown as described previously[1-3]. Following agroinfiltration, tobacco plants were transferred to climate chambers with 21°C/19°C during 12/12 hours day/night periods and 70% relative humidity.

Production and purification of recombinant effector proteins

The sequences encoding mature VdAve1, VnAve1, and XacPNP were amplified from pGEM-T plasmid DNA carrying the respective gene[4]. Forward and reverse primers included *Xho*I and *Bam*HI restriction sites, respectively, for restriction digest and ligation into pET-15b with an N-terminal His_6 tag sequence (Novagen, Madison, WI, USA) (Supplementary Table 1). The resulting expression vectors were confirmed by sequencing and used to transform *E. coli* strain BL21. Positive transformants were selected by colony PCR using a pFBT005 forward primer with gene specific reverse primers (Supplementary Table 1).

For heterologous protein production, BL21 cells were grown in 1xYT liquid medium at 37°C with constant shaking at 200 rpm. Protein production was induced with 1 mM IPTG final concentration when cultures reached an OD_{600}=2 to ensure maximum yields. To favor the generation of inclusion bodies, cultures were kept at 42°C, 200 rpm for 2 hours of protein production. Cell pellets were snap-frozen in liquid nitrogen and then washed with 100 mM NaCl, 1 mM EDTA, and 10 mM Tris at pH 8.5. Cells were disrupted by stirring for 1 hour in lysis buffer (100 mM Tris, 150 mM NaCl, 10% glycerol, 6 mg/mL lysozyme (Sigma, St. Louis, MO, USA), 2 mg/mL deoxycholic acid, 0.06 mg/mL DNaseI, protease inhibitor cocktail (Roche, Mannheim, Germany)) at 4°C. Soluble and insoluble fractions were separated by centrifuging at 20,000 x *g* for 10 min. The insoluble protein pellets were washed with 10 mL 1 M guanidine hydrochloride (GnHCl), 10 mM Tris at pH 8.0 and then denatured in 10 mL 6 M GnHCl, 10 mM β-mercaptoethanol, 10 mM Tris at pH 8.0. Samples were incubated for 1 hour at room temperature. Non-denatured debris was pelleted by centrifuging at 20,000 x *g* for 10 min and discarded. Denaturation was allowed to continue for additional 3-4 hours.

Heterologously produced proteins were then purified by metal affinity chromatography using a column packed with 10 mL 50% His60 Ni^{2+} Superflow Resin (Clontech, Mountain View, CA, USA) and connected to a BioLogic LP System (BioRad, Veenendaal, The Netherlands). Each run was monitored with the LP Data View software (V1.03; BioRad, Veenendaal, The Netherlands). The column was equilibrated with 5-10 volumes of wash buffer (6 M GnHCl, 10 mM Tris, 20 mM imidazole, 10 mM reduced glutathione, 2 mM

oxidized glutathione, pH 8.0). Protein samples were loaded at a flow rate of 0.5 mL/min to allow maximum binding of affinity-tagged proteins to the nickel resin. Contaminating proteins were removed by washing with 10-20 column volumes of washing buffer. Bound proteins were either eluted in a 50 mL gradient from 100% washing buffer to 100% elution buffer (6 M GnHCl, 10 mM Tris, 200 mM NaCl, 500 mM imidazole, 10 mM reduced glutathione, 2 mM oxidized glutathione, pH 8.0) or under denaturing conditions. Eluted fractions containing recombinant proteins were analyzed by PAGE separation on 4-15% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (BioRad, Veenendaal, The Netherlands). Fractions containing the protein of interest were pooled. Recombinant proteins eluted in denaturation buffer were dialyzed (Spectra/Por®3 Dialysis Membrane, MWCO= 3.5 kDa) step-wise against 20 volumes of 0.25 M ammonium sulfate, 0.1 M BisTris, pH 5.5 with decreasing GnHCl concentrations for refolding. Each dialysis step was allowed to proceed for at least 2 hours. Samples were kept overnight in buffer containing 1 M GnHCl supplemented with glutathione to allow further disulphide reshuffling. For downstream applications, proteins were then dialyzed against 0.25 M ammonium sulfate, 0.1 M BisTris, pH 5.5 and concentrated over Amicon Ultra Centrifugal units (MWCO= 3 kDa, Merck Millipore, Cork, Ireland). Final concentrations were determined using the BioRad Protein Assay (BioRad, Veenendaal, The Netherlands).

Biomass quantification

For *V. dahliae* biomass quantification total DNA was extracted from lyophilized tomato stem sections of infected plants using a CTAB-based extraction buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA, 2 M NaCl, 3% CTAB). DNA was precipitated with 1.5 M ammonium acetate and absolute ethanol. Quantitative RT-PCR was carried out with the ITS1_F/St-Ve1_R primer pair (Supplementary Table 1). For sample calibration, the tomato Rubisco gene (*SlRUB*) was targeted using *SlRUB* F and R primers (Supplementary Table 1). Quantitative RT-PCR conditions and result analyses were as described elsewhere[5].

VdAve1 target identification by immunopurification and mass spectrometry

Immunopurifications were carried out as described previously[6] with minor modifications. N-terminally tagged GFP-Ave1[4] and Ve1-HA[3] were produced in fully expanded leaves of three or four 5 to 6 week-old *N. tabacum* cv. Petit Havana SR1 plants. Total proteins were extracted using extraction buffer (EB) (50 mM Tris, 150 mM NaCl, 1% IGEPAL CA-630 (NP40), protease inhibitor cocktail (Roche), pH 8.0). For immunopurification, 2 mg of total protein were incubated with 25 µL 50% slurry of GFP-Trap®_A beads (Chromotek, Planegg-Martinsried, Germany) or α-HA affinity matrix (Pierce, Rockford, IL, USA) shaking for 1 hour at 4°C. Beads were washed five times with EB. Peptides generated by tryptic on-bead digestion of immunopurified proteins were analyzed using a Proxeon EASY nanoLC

connected to a Thermo LTQ-Orbitrap XL mass spectrometer[7]. For protein identification, the publicly available tobacco (*N. tabacum*) database from Uniprot (2013) and an in-house *V. dahliae* (PeterEsse_JR2_Genome_2013) databases were queried. Results were analysed with MaxQuant 1.3.0.5. in label-free mode[8]. Result filtering and statistical analyses were carried out as described previously[9].

Chitinase expression in infected tomato plants

Tomato stem sections were collected from 9 plants 4, 8, and 12 days after inoculation with wild-type *V. dahliae* or water as mock treatment. Samples of three plants were pooled for each treatment and time point. Chitinase expression was analyzed using previously published primer pairs[10]. Quantitative RT-PCR was carried out as previously[5]. Expression values of infected plants were normalized to those in mock-treated tissue.

Purification of recombinant tomato chitinases

Following fermentation, cell-free supernatants were concentrated using a Vivaflow 200 crossflow device (MWCO= 5 kDa, Sartorius, Göttingen, Germany) and purified under native conditions by metal affinity chromatography using a column packed with 10 mL 50% His60 Ni^{2+} Superflow Resin (Clontech, Mountain View, CA, USA) and connected to a BioLogic LP Sytem (BioRad, Veenendaal, The Netherlands). Each run was monitored with the LP Data View software (Version 1.03; BioRad, Veenendaal, The Netherlands). The column was equilibrated with 5-10 volumes of washing buffer (20 mM Tris, 200 mM NaCl, 20 mM imidazole, pH 8.0). After sample loading, the column was washed with 20 column volumes of washing buffer to remove contaminants. Specifically bound proteins were eluted in a 50 mL gradient from 100% washing buffer to 100% elution buffer (20 mM Tris, 200 mM NaCl, 500 mM imidazole, pH 8.0). Fractions containing recombinant chitinase proteins were combined and dialyzed (Spectra/Por®3 Dialysis Membrane; MWCO= 3.5 kDa) against 100 volumes of 200 mM NaCl overnight. Samples were concentrated and concentrations measured as described above.

References supplementary methods

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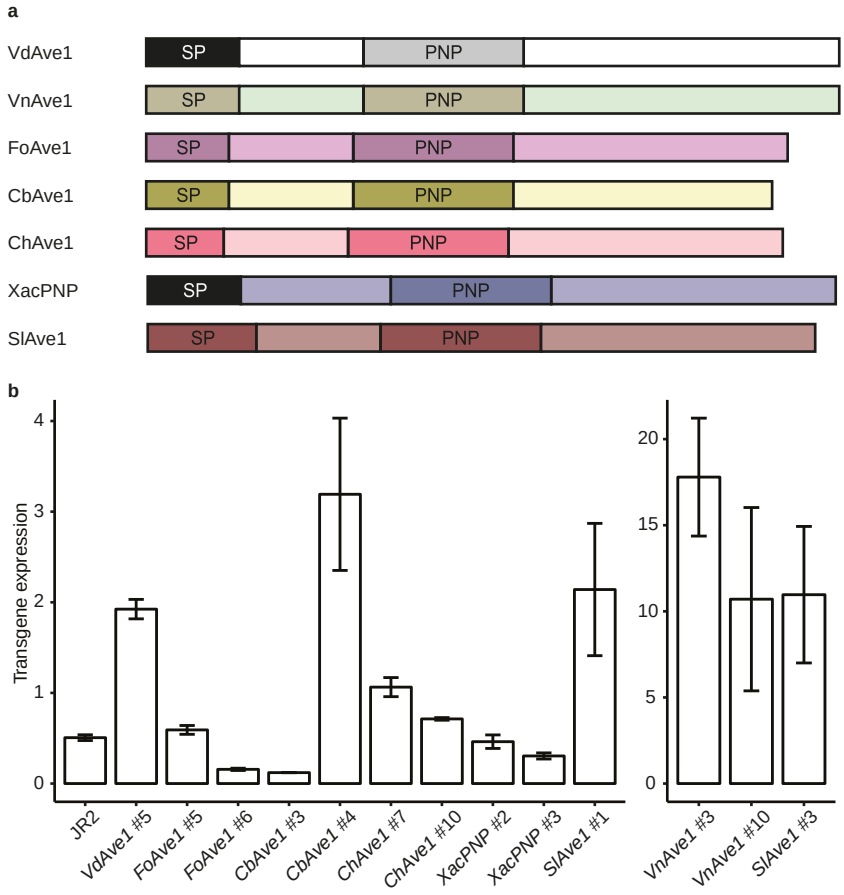
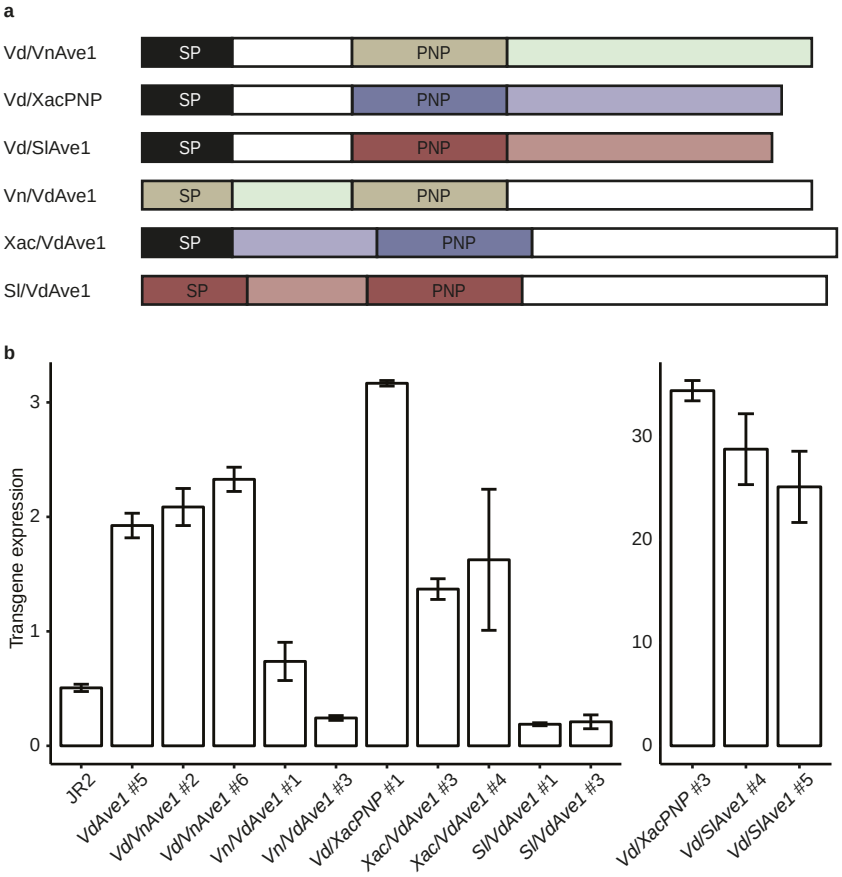
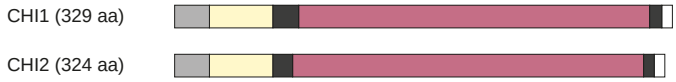
Supplementary data



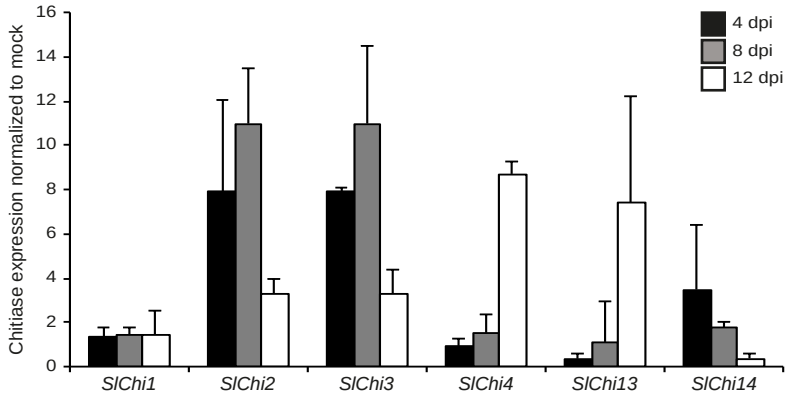
SUPPLEMENTARY FIGURE 1 | Construct design and expression of *VdAve1* homologous genes from microbes and tomato in *V. dahliae*. **a**, Schematic representation of VdAve1 homologs as expressed in the *VdAve1* deletion mutant. The native signal peptide of the bacterial homolog XacPNP was replaced with that of VdAve1 to ensure secretion by *V. dahliae*. **b**, *In vitro* expression of transgenes in selected strains. Following selection, transformants were grown in liquid ½ MS medium for 5 days. Transgene expression levels were determined by qRT-PCR using transgene-specific primers and *VdGAPDH* for sample calibration. Bars represent mean expression values of 2-3 independently grown cultures (only *CbAve1* #3 was grown once) ± SD.



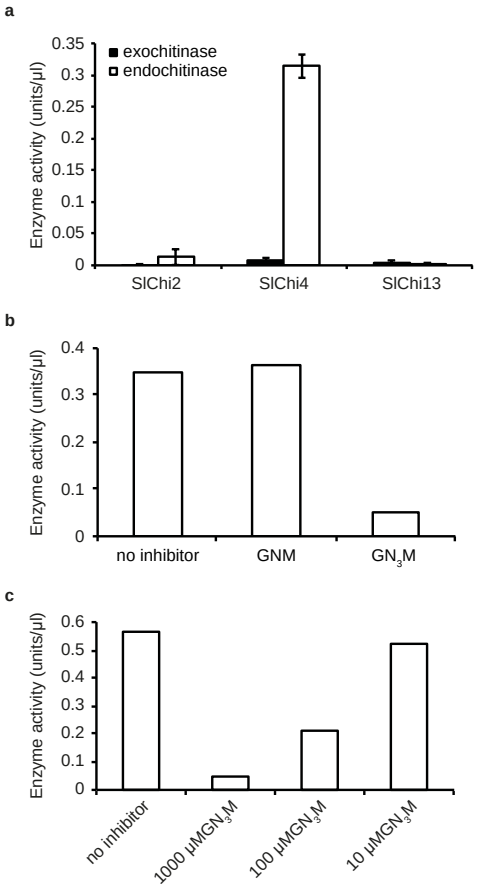
SUPPLEMENTARY FIGURE 2 | Construct design and expression of chimeric *Ave1* mutants. a, Schematic representation of Ave1 chimeras as expressed in the *VdAve1* deletion mutant. Black and white boxes represent domains originating from VdAve1. Colored boxes show domains taken from homologs. **b**, *In vitro* expression of transgenes in selected strains. Transgene expression levels were determined by qRT-PCR using transgene-specific primers and *VdGAPDH* for sample calibration. Bars represent mean expression values of 2-3 independently grown cultures ± SD.



SUPPLEMENTARY FIGURE 3 | Graphic representation of tobacco chitinases. Protein structures of tobacco CHI1 and CHI2 include signal peptides (light grey), chitin-binding type-1 domains (yellow), glycosyl hydrolase 19 domains (red), and a pro-peptide at the C-terminus (white), which is removed in the mature form of each protein.



SUPPLEMENTARY FIGURE 4 | Expression of tomato endochitinase genes during *V. dahliae* infection. Tomato stem tissue was harvested at 4, 8, and 12 days following inoculation with wild type *V. dahliae*. Endochitinase expression levels were determined by qRT-PCR with chitinase gene-specific primers and primers targeting the tomato tubulin gene for calibration. Shown are expression values relative to mock-treated tissue of three biological replicates consisting of pools of three plants from a single experiment (mean ± SD). This experiment was performed twice.



SUPPLEMENTARY FIGURE 5 | The chitinase inhibitor GN_3M efficiently inhibits tomato endochitinase SlChi4. a, Comparison of chitinase activities. SlChi2, SlChi4, and SlChi13 (0.5 µM) were incubated with 0.2 mg/mL substrates for 30 min at 37°C. The highest amount of *p*-nitrophenol product was measured following endochitinolytic cleavage by SlChi4. Hardly any activity was detected for SlChi2 and SlChi13. Bars show mean values from two independent experiments with two technical replicates ± SD. **b**, Moranoline-coupled chitotriose (GN_3M) suppresses SlChi4 activity. GN_3M and GNM were added to SlChi4 at final concentrations of 1000 µM. Following pre-incubation at room temperature, the mixtures were added to the substrate in reaction buffer. Enzyme activity was measured after incubation of 30 min at 37°C. Data are from a single experiment. The experiment was performed twice. **c**, In order to determine whether the inhibitory effect of GN_3M on SlChi4 is concentration-dependent, SlChi4 was pre-incubated with serial dilutions of GN_3M. Chitinase activity was determined as before. Data are from a single experiment. The experiment was performed twice.

SUPPLEMENTARY TABLE 1 | Primers used in this study.

Name	Sequence (5' → 3')	Application
*Xho*I_VdAve1_F	CGGTATCTCGAGGATCTAGGGACCGCATCCTAC	Protein production
VdAve1_*Bam*HI_R	CGTCTAGGATCCTCATTATTATATCTGTCTAAATTCGATGTTGACC	Protein production
*Xho*I_VnAve1_F	CGGTATCTCGAGCAATTAGGGACCGCATCC	Protein production
VnAve1_*Bam*HI_R	CGTCTAGGATCCTCATTATTATATCTGTTCAAACTCG	Protein production
*Xho*I_XacPNP_F	CGGTATCTCGAGGACATCGGTACAATTAG	Protein production
XacPNP_*Bam*HI_R	CGTCTAGGATCCTCATTATTAAATATTTGCCCAGGG	Protein production
pFBT005_F	GCCAGACCAATACAACAAGCA	Colony PCR and sequencing
pFBT005_R	TCGAGATCCTGAACACCATTT	Sequencing
VdAve1_qPCR_F	TGTTACCAAAGCAGCACACAAGG	Real-time PCR
VdAve1_qPCR_R	CCTTATGCCTCGTTCCCTTCCAC	Real-time and colony PCR
VnAve1_qPCR_F	GAGCCCAGACAGCTGTCAC	Real-time PCR
VnAve1_qPCR_R	TTCAAACTCGATGTTGACCTTCT	Real-time and colony PCR
FoAve1_qPCR_F	ATATCGGAACTGCAAATATTCTCAAC	Real-time PCR
FoAve1_qPCR_R	CTTATACATTTCATCGTATACAGTCTGC	Real-time PCR
CbAve1_qPCR_F	ATTCCCTTCAGGCAACCTCT	Real-time PCR
CbAve1_qPCR_R	CGGACAAGCTTCGCAATAAT	Real-time PCR
ChAve1_qPCR_F	CAAGATGCTATGGCAACAATATGAAC	Real-time PCR
ChAve1_qPCR_F	GTCTTGAGGAAAATCTATCGTATTTCTG	Real-time PCR
XacPNP_qPCR_F	GCAATCGGTTTGCTCTTTTC	Real-time PCR
XacPNP_qPCR_R	AGCACCGTTATCCCACAGAC	Real-time and colony PCR
SlAve1_qPCR_F	CGTCGGGGAATCTATTTGTG	Real-time PCR
SlAve1_qPCR_R	AAAGCATCCGTTGACAAAGC	Real-time PCR
VdGAPDH_F	CGAGTCCACTGGTGTCTTCA	Real-time and colony PCR
VdGAPDH_R	CCCTCAACGATGGTGAACTT	Real-time and colony PCR
ITS1-F	AAAGTTTTAATGGTTCGCTAAGA	Real-time PCR
St-Ve1-R	CTTGGTCATTTAGAGGAAGTAA	Real-time PCR
SlRUB_F	GAACAGTTTCTCACTGTTGAC	Real-time PCR
SlRUB_R	CGTGAGAACCATAAGTCACC	Real-time PCR
SlTUB_F	AACCTCCATTCAGGAGATGTTT	Real-time PCR
SlTUB_R	TCTGCTGTAGCATCCTGGTATT	Real-time PCR
SlChi_F(TRV)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTTGCCCAAACTTCCCA	VIGS
SlChi_R(TRV)	GGGGACCACTTTGTACAAGAAAGCTGGGTACTGCCGTGACCACATTCCA	VIGS
SlChi2_F	ATGAGGCTTTCTGAATTCAC	coIP
SlChi2-HA_R	CTAAGCGTAGTCTGGGACGTCGTATGGGTACATAATATCAACTAATAGTC	coIP
SlChi4_F	ATGAGGCATTTTGAATTCATAG	coIP
SlChi4-HA_R	CTAAGCGTAGTCTGGGACGTCGTATGGGTACATAGTATCGACTAAGAGTCCG	coIP
attB1-SlChi2_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAGGCTTTCTGAATTCAC	coIP
attB1-SlChi4_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAGGCATTTTGAATTCATAG	coIP
HA-attB2_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAAGCGTAGTCTGGGACG	coIP

SUPPLEMENTARY TABLE 2 | Proteins identified by mass spectrometry.

LOG protein abundance ratio (GFP-VdAve1/ Ve1-HA	LOG average iBAQ	Significance	Fasta headers
4.995973349	6.490961081	significant	>sp|P42212|GFP_AEQVI Green fluorescent protein OS=Aequorea victoria GN=GFP PE=1 SV=1;>sp|P42212mut|YFP (GFP mutant)
4.649643421	4.710973932	significant	>VDJR2_2804_eg45-like domain containing protein_Ave1
3.105216742	6.154132156	significant	>tr|Q7M242|Q7M242_TOBAC Glutamate synthase (Ferredoxin) (Clone C(35)) (Fragment) OS=Nicotiana tabacum PE=4 SV=1
3.030462384	5.686786516	significant	>tr|Q6QND3|Q6QND3_TOBAC Putative pyridoxine biosynthesis protein isoform A OS=Nicotiana tabacum GN=Pdx1-B PE=3 SV=1
2.797689199	3.305277801	significant	>sp|P08252|CHI1_TOBAC Endochitinase A OS=Nicotiana tabacum GN=CHN48 PE=1 SV=2;>sp|P24091|CHI2_TOBAC Endochitinase B OS=Nicotiana tabacum GN=CHN50 PE=1 SV=1
2.794015408	6.611454274	significant	>sp|P41919|RANB1_TOBAC GTP-binding nuclear protein Ran-B1 OS=Nicotiana tabacum GN=RAN-B1 PE=2 SV=1
2.73314786	6.3426939	significant	>tr|Q9FXS7|Q9FXS7_TOBAC EIG-I24 protein OS=Nicotiana tabacum GN=EIG-I24 PE=2 SV=1
2.586536288	5.981394195	significant	>tr|Q7X998|Q7X998_TOBAC MRNA-binding protein (Fragment) OS=Nicotiana tabacum GN=csp41 PE=2 SV=1
2.438526034	1.280666012	non-significant	>sp|P30361|UCRIA_TOBAC Cytochrome b6-f complex iron-sulfur subunit 1, chloroplastic OS=Nicotiana tabacum GN=petC1 PE=2 SV=2;>sp|Q02585|UCRIB_TOBAC Cytochrome b6-f complex iron-sulfur subunit 2, chloroplastic OS=Nicotiana tabacum GN=petC2 PE=2 SV=1
2.424926162	4.222165304	significant	>tr|Q3LAG6|Q3LAG6_TOBAC Cysteine synthase (Fragment) OS=Nicotiana tabacum GN=oas1 PE=2 SV=1
2.385375857	7.297352975	significant	>tr|Q40480|Q40480_TOBAC C-7 protein OS=Nicotiana tabacum GN=C-7 PE=2 SV=1
2.301433484	1.355083518	non-significant	>tr|Q40589|Q40589_TOBAC Cytosolic ascorbate peroxidase OS=Nicotiana tabacum PE=2 SV=1;>tr|Q42941|Q42941_TOBAC Ascorbate peroxidase OS=Nicotiana tabacum GN=APX PE=2 SV=1;>tr|Q8W1K9|Q8W1K9_TOBAC Ascorbate peroxidase (Fragment) OS=Nicotiana tabacum PE=2 SV=1
2.273143609	1.084217929	non-significant	>tr|O24511|O24511_TOBAC Catalase OS=Nicotiana tabacum GN=cat1 PE=2 SV=1
2.159630299	5.685583504	significant	>tr|O24135|O24135_TOBAC Citrate synthase OS=Nicotiana tabacum GN=cit1 PE=2 SV=1e
2.129659891	4.716856896	significant	>tr|Q5XMB8|Q5XMB8_TOBAC Cytosolic acetoacetyl-coenzyme A thiolase OS=Nicotiana tabacum GN=AACT1 PE=2 SV=1
2.109525124	1.287513182	non-significant	>tr|Q8RVF8|Q8RVF8_TOBAC Thioredoxin peroxidase OS=Nicotiana tabacum PE=2 SV=2;>tr|K0I7G7|K0I7G7_TOBAC Thioredoxin peroxidase (Fragment) OS=Nicotiana

LOG protein abundance ratio (GFP-VdAve1/ Ve1-HA	LOG average iBAQ	Significance	Fasta headers
2.054849704	1.01706741	non-significant	>tr|Q04106|Q04106_TOBAC Prb-1b OS=Nicotiana tabacum GN=PRB-1B PE=4 SV=1
2.030626098	1.342591934	non-significant	>tr|Q9LEB0|Q9LEB0_TOBAC Pectinesterase OS=Nicotiana tabacum PE=2 SV=1;>tr|Q9SC79|Q9SC79_TOBAC Pectinesterase (Fragment) OS=Nicotiana tabacum GN=pectin methylesterase PE=2 SV=1
1.962767482	4.680847563	significant	>tr|O65855|O65855_TOBAC Glucose-6-phosphate 1-dehydrogenase OS=Nicotiana tabacum PE=2 SV=1;>tr|O65854|O65854_TOBAC Glucose-6-phosphate 1-dehydrogenase OS=Nicotiana tabacum PE=2 SV=1
1.937334736	1.210653414	non-significant	>tr|Q9FEL3|Q9FEL3_TOBAC Elongation factor 2 (Fragment) OS=Nicotiana tabacum GN=ef2 PE=2 SV=1
1.930932681	1.09982392	non-significant	>tr|O82077|O82077_TOBAC Glycolate oxidase (Fragment) OS=Nicotiana tabacum GN=GLO PE=4 SV=1
1.836010933	1.042393256	non-significant	>sp|P25871|OLPA_TOBAC Osmotin-like protein OS=Nicotiana tabacum GN=OLPA PE=1 SV=1;>tr|Q75W83|Q75W83_TOBAC Pathogenesis-related protein (Fragment) OS=Nicotiana tabacum GN=PR-5dA PE=2 SV=1;>tr|Q208P5|Q208P5_TOBAC Osm
1.793446223	1.136020593	non-significant	>sp|P09043|G3PA_TOBAC Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic (Fragment) OS=Nicotiana tabacum GN=GAPA PE=2 SV=1
1.785473466	5.538226112	significant	>tr|Q40501|Q40501_TOBAC 37kDa chloroplast inner envelope membrane polypeptide OS=Nicotiana tabacum PE=2 SV=1
1.756061673	1.037479542	non-significant	>tr|Q53UI6|Q53UI6_TOBAC PsbQ OS=Nicotiana tabacum GN=psbQ PE=2 SV=1;>tr|Q5EFR4|Q5EFR4_TOBAC Chloroplast oxygen-evolving protein 16 kDa subunit OS=Nicotiana tabacum GN=psbQ PE=2 SV=1
1.753452222	0.76407689	non-significant	>sp|P43643|EF1A_TOBAC Elongation factor 1-alpha OS=Nicotiana tabacum PE=2 SV=1;>tr|P93769|P93769_TOBAC Elongation factor 1-alpha OS=Nicotiana tabacum GN=tel1 PE=2 SV=1
1.742637753	1.243388774	non-significant	>tr|Q43799|Q43799_TOBAC Beta-fructosidase (Beta-fructofuranosidase) OS=Nicotiana tabacum GN=Ntbfruc1 PE=2 SV=1;>tr|Q8W3Z9|Q8W3Z9_TOBAC Invertase (Fragment) OS=Nicotiana tabacum GN=NtINV PE=2 SV=1
1.732776483	1.327666567	non-significant	>sp|O22436|CHLI_TOBAC Magnesium-chelatase subunit ChlI, chloroplastic OS=Nicotiana tabacum GN=CHLI PE=2 SV=1;>tr|Q9FES7|Q9FES7_TOBAC Sulfur OS=Nicotiana tabacum GN=Su-s PE=2 SV=1;>tr|Q708F6|Q708F6_T
1.696649909	1.188624794	non-significant	>tr|Q50LG4|Q50LG4_TOBAC Peroxidase OS=Nicotiana tabacum GN=NtPOX2 PE=2 SV=1;>tr|Q50LG5|Q50LG5_TOBAC Peroxidase OS=Nicotiana tabacum GN=NtPOX1 PE=2 SV=1
1.695626616	1.2810669	non-significant	>sp|P62094|PSAC_TOBAC Photosystem I iron-sulfur center OS=Nicotiana tabacum GN=psaC PE=1 SV=2

LOG protein abundance ratio (GFP-VdAve1/ Ve1-HA	LOG average iBAQ	Significance	Fasta headers
1.689821283	0.72171936	non-significant	>tr|B5APL3|B5APL3_TOBAC Basic beta-1,3-glucanase OS=Nicotiana tabacum PE=3 SV=1;>sp|P23546|E13E_TOBAC Glucan endo-1,3-beta-glucosidase, basic vacuolar isoform GGIB50 OS=Nicotiana tabacum PE=1 SV=1
1.631613533	1.190498154	non-significant	>tr|Q8H1T6|Q8H1T6_TOBAC Phospholipase D beta 1 isoform (Fragment) OS=Nicotiana tabacum PE=2 SV=1;>tr|Q9SNY2|Q9SNY2_TOBAC Phospholipase D beta 1 isoform (Fragment) OS=Nicotiana tabacum PE=2 SV=2
1.593666236	0.958177393	non-significant	>tr|D6PZY5|D6PZY5_TOBAC RNA-binding glycine-rich protein OS=Nicotiana tabacum GN=RGP-1a PE=2 SV=1
1.564302564	0.567137691	non-significant	>sp|P08299|PR1A_TOBAC Pathogenesis-related protein 1A OS=Nicotiana tabacum PE=1 SV=1;>tr|Q53WU3|Q53WU3_TOBAC Tobacco PR-1a pathogenesis-related protein 1a OS=Nicotiana tabacum PE=4 SV=1;>sp|P07053|PR1B_TOBAC Pathogenesis-related protein 1B OS=Nicotiana ta
1.495542288	0.893273874	non-significant	>tr|Q41231|Q41231_TOBAC Pathogen-and wound-inducible antifungal protein CBP20 OS=Nicotiana tabacum GN=CBP20 PE=2 SV=1;>tr|Q41230|Q41230_TOBAC CBP20 (Fragment) OS=Nicotiana tabacum GN=CBP20 PE=2 SV=1
1.477790634	0.733172375	non-significant	>sp|Q04127|PSBP3_TOBAC Oxygen-evolving enhancer protein 2-3, chloroplastic OS=Nicotiana tabacum GN=PSBP3 PE=2 SV=1
1.4440732	1.001784284	non-significant	>tr|Q9FSF0|Q9FSF0_TOBAC Malate dehydrogenase OS=Nicotiana tabacum GN=md1 PE=2 SV=1
1.414322456	1.11105724	non-significant	>tr|H9CCH6|H9CCH6_TOBAC Acetyl-CoA carboxylase OS=Nicotiana tabacum GN=ACC PE=2 SV=1;>tr|Q40475|Q40475_TOBAC Biotin carboxylase subunit OS=Nicotiana tabacum PE=2 SV=1
1.395544291	1.281187192	non-significant	>tr|Q8SF04|Q8SF04_TOBAC Dicarboxylate/tricarboxylate carrier OS=Nicotiana tabacum GN=dtc1 PE=2 SV=1;>tr|Q9FSF4|Q9FSF4_TOBAC Mitochondrial 2-oxoglutarate/malate carrier protein OS=Nicotiana tabacum GN=momc1 PE=2 SV=1;>tr|Q8SF03|Q8SF03_TOBAC Dicarboxylate/t
1.391538779	0.937437417	non-significant	>tr|O81102|O81102_TOBAC Glutamate decarboxylase isozyme 1 OS=Nicotiana tabacum GN=NtGAD1 PE=2 SV=1;>tr|P93369|P93369_TOBAC Glutamate decarboxylase OS=Nicotiana tabacum GN=NtGAD1 PE=2 SV=1;>tr|Q9AT17|Q9AT17_TOBAC Glutamate decarboxylase isozyme 1 OS=Nicoti
1.374907136	0.577433107	non-significant	>sp|P27492|CB21_TOBAC Chlorophyll a-b binding protein 16, chloroplastic OS=Nicotiana tabacum GN=CAB16 PE=2 SV=1;>sp|P27491|CB27_TOBAC Chlorophyll a-b binding protein 7, chloroplastic OS=Nicotiana tabacum GN=CAB7 PE=3 SV=1;>sp|P27496|CB25_TOBAC Chlorophyll
1.313286026	0.646669285	non-significant	>sp|P29790|ATPG_TOBAC ATP synthase gamma chain, chloroplastic OS=Nicotiana tabacum GN=ATPC PE=1 SV=1

LOG protein abundance ratio (GFP-VdAve1/ Ve1-HA	LOG average iBAQ	Significance	Fasta headers
1.258415262	0.743014617	non-significant	>sp|O82702|VATG1_TOBAC V-type proton ATPase subunit G 1 OS=Nicotiana tabacum GN=VATG1 PE=3 SV=1
1.251875202	0.788416019	non-significant	>sp|P32980|ATPD_TOBAC ATP synthase delta chain, chloroplastic OS=Nicotiana tabacum GN=ATPD PE=2 SV=1
1.23220706	1.244382767	non-significant	>sp|P06363|RR8_TOBAC 30S ribosomal protein S8, chloroplastic OS=Nicotiana tabacum GN=rps8 PE=3 SV=1
1.164635658	0.689133602	non-significant	>tr|Q04126|Q04126_TOBAC 23-kDa ploypeptide of photosystem II oxygen-evolving complex OS=Nicotiana tabacum GN=oee2-B PE=2 SV=1
1.163202167	0.692201194	non-significant	>tr|A1KYB0|A1KYB0_TOBAC Adenosylhomocysteinase OS=Nicotiana tabacum GN=SAHH2 PE=2 SV=1;>tr|A1KYB2|A1KYB2_TOBAC Adenosylhomocysteinase OS=Nicotiana tabacum GN=SAHH4 PE=2 SV=1;>sp|P68173|SAHH_TOBAC Adenosylhomocysteinase OS=Nicotiana tabacum GN=SAHH PE=2 SV
1.1475209	0.574872384	non-significant	>sp|P14170|OSMO_TOBAC Osmotin OS=Nicotiana tabacum GN=AP24 PE=1 SV=2
1.139393091	0.684645548	non-significant	>sp|Q7DM39|PSBP1_TOBAC Oxygen-evolving enhancer protein 2-1, chloroplastic OS=Nicotiana tabacum GN=PSBP1 PE=3 SV=2;>tr|Q40457|Q40457_TOBAC 23 kDa polypeptide of water-oxidizing complex of photosystem II OS=Nicotiana tabacum PE=2 SV=1
1.138918718	0.505099322	non-significant	>sp|Q40460|RCA1_TOBAC Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplastic OS=Nicotiana tabacum PE=1 SV=1
1.108122627	0.639913216	non-significant	>sp|P41381|IF4A8_TOBAC Eukaryotic initiation factor 4A-8 OS=Nicotiana tabacum PE=2 SV=1;>sp|P41382|IF410_TOBAC Eukaryotic initiation factor 4A-10 OS=Nicotiana tabacum PE=2 SV=1;>sp|Q40465|IF411_TOBAC Eukaryotic initiation factor 4A-11 OS=Nicotiana tabacum
1.088739991	0.571831378	non-significant	>sp|P06290|ATPF_TOBAC ATP synthase subunit b, chloroplastic OS=Nicotiana tabacum GN=atpF PE=2 SV=3;>tr|Q36600|Q36600_TOBAC ATPase subunit I OS=Nicotiana tabacum PE=3 SV=1
1.070559859	0.692129107	non-significant	>tr|Q5DKU9|Q5DKU9_TOBAC Adenosine kinase isoform 1S OS=Nicotiana tabacum PE=2 SV=1
1.069312334	0.691663994	non-significant	>tr|P93358|P93358_TOBAC Protein disulfide-isomerase OS=Nicotiana tabacum GN=PDI PE=2 SV=1
1.054344734	0.424240653	non-significant	>tr|Q9FEL2|Q9FEL2_TOBAC Elongation factor 2 (Fragment) OS=Nicotiana tabacum GN=ef2 PE=2 SV=1
1.051309705	0.692178971	non-significant	>tr|Q9AVG8|Q9AVG8_TOBAC Isopentenyl diphosphate isomerase 1 OS=Nicotiana tabacum GN=ipi1 PE=2 SV=1;>tr|Q9AVG7|Q9AVG7_TOBAC Isopentenyl diphosphate isomerase 2 OS=Nicotiana tabacum GN=ipi2 PE=2 SV=1

LOG protein abundance ratio (GFP-VdAve1/ Ve1-HA	LOG average iBAQ	Significance	Fasta headers
1.051091711	0.518069558	non-significant	>sp|P06407|PSAB_TOBAC Photosystem I P700 chlorophyll a apoprotein A2 OS=Nicotiana tabacum GN=psaB PE=3 SV=1;>tr|Q15B79|Q15B79_TOBAC PSI P700 apoprotein A2 OS=Nicotiana tabacum GN=psaB PE=4 SV=1
1.033475478	0.459097172	non-significant	>tr|B0FPA4|B0FPA4_TOBAC Extracellular Ca2+ sensing receptor OS=Nicotiana tabacum GN=cas PE=2 SV=1
1.011196017	0.690934206	non-significant	>tr|Q43583|Q43583_TOBAC Hsr201 protein OS=Nicotiana tabacum GN=hsr201 PE=2 SV=1;>sp|Q8GT20|BEBT_TOBAC Benzyl alcohol O-benzoyltransferase OS=Nicotiana tabacum GN=HSR201 PE=1 SV=1
0.996478279	0.945717179	non-significant	>sp|Q42962|PGKY_TOBAC Phosphoglycerate kinase, cytosolic OS=Nicotiana tabacum PE=2 SV=1
0.99031647	0.557915895	non-significant	>tr|Q3LAG5|Q3LAG5_TOBAC Cysteine synthase OS=Nicotiana tabacum GN=oas7 PE=2 SV=1
0.989691099	0.420754711	non-significant	>sp|P69556|PSBA_TOBAC Photosystem Q(B) protein OS=Nicotiana tabacum GN=psbA PE=3 SV=2
0.941258073	0.549436626	non-significant	>sp|P00834|ATPE_TOBAC ATP synthase epsilon chain, chloroplastic OS=Nicotiana tabacum GN=atpE PE=3 SV=1
0.936209242	0.573518764	non-significant	>tr|D2K7Z2|D2K7Z2_TOBAC Photosystem I reaction center subunit OS=Nicotiana tabacum GN=PsaN PE=2 SV=1
0.932843606	0.780645982	non-significant	>tr|A7XAQ5|A7XAQ5_TOBAC Glucose-1-phosphate adenylyltransferase OS=Nicotiana tabacum GN=AGP PE=2 SV=1
0.929069281	0.584744087	non-significant	>tr|Q76ME6|Q76ME6_TOBAC Calmodulin NtCaM10 OS=Nicotiana tabacum GN=NtCaM10 PE=2 SV=1;>tr|Q76MF3|Q76MF3_TOBAC Calmodulin NtCaM11 OS=Nicotiana tabacum GN=NtCaM3 PE=2 SV=1
0.922771096	0.45315415	non-significant	>tr|E9NZ46|E9NZ46_TOBAC Molecular chaperone Hsp90 OS=Nicotiana tabacum PE=2 SV=1;>tr|Q14TB1|Q14TB1_TOBAC Heat shock protein 90 OS=Nicotiana tabacum GN=hsp90 PE=2 SV=1;>tr|Q6R0J1|Q6R0J1_TOBAC Heat shock protein 90 OS=Nicotiana tabacum GN=OINtHsp90 PE=2 SV=
0.868075848	0.385171553	non-significant	>tr|Q84QE7|Q84QE7_TOBAC Putative photosystem I subunit III OS=Nicotiana tabacum PE=2 SV=1
0.843497594	3.496778233	non-significant	>tr|F2VJ75|F2VJ75_TOBAC Fructose-bisphosphate aldolase OS=Nicotiana tabacum PE=2 SV=1
0.814845403	0.29015083	non-significant	>tr|B2YKT9|B2YKT9_TOBAC Glycine-rich RNA-binding protein OS=Nicotiana tabacum GN=GRP1 PE=2 SV=1
0.759289265	0.674918295	non-significant	>tr|O65852|O65852_TOBAC Isocitrate dehydrogenase (NAD+) OS=Nicotiana tabacum PE=2 SV=1

LOG protein abundance ratio (GFP-VdAve1/ Ve1-HA	LOG average iBAQ	Significance	Fasta headers
0.749608517	0.34082002	non-significant	>sp|Q40459|PSBO_TOBAC Oxygen-evolving enhancer protein 1, chloroplastic OS=Nicotiana tabacum GN=PSBO PE=2 SV=1
0.688959956	0.445998084	non-significant	>tr|Q42958|Q42958_TOBAC Catechol O-methyltransferase OS=Nicotiana tabacum GN=OMT I-a PE=2 SV=1
0.688061396	0.45433902	non-significant	>sp|Q9ZS34|CHLP_TOBAC Geranylgeranyl diphosphate reductase, chloroplastic OS=Nicotiana tabacum GN=CHLP PE=2 SV=1
0.666500966	1.520130861	non-significant	>sp|P49319|CATA1_TOBAC Catalase isozyme 1 OS=Nicotiana tabacum GN=CAT-1 PE=1 SV=2
0.660817464	0.246114173	non-significant	>tr|Q0PWS7|Q0PWS7_TOBAC Chloroplast pigment-binding protein CP29 OS=Nicotiana tabacum GN=Lhcb4 PE=2 SV=1
0.508342028	0.799660982	non-significant	>tr|Q67BD0|Q67BD0_TOBAC Heat shock protein 70-3 OS=Nicotiana tabacum GN=HSP70-3 PE=3 SV=1;>tr|Q84QJ3|Q84QJ3_TOBAC Heat shock protein 70 OS=Nicotiana tabacum GN=Hsp70 PE=2 SV=1
0.437040448	0.142610187	non-significant	>tr|Q8LNZ4|Q8LNZ4_TOBAC Tobacco nucleolin OS=Nicotiana tabacum GN=NNF1 PE=2 SV=1
0.434017142	1.828117234	non-significant	>sp|P00826|ATPB_TOBAC ATP synthase subunit beta, chloroplastic OS=Nicotiana tabacum GN=atpB PE=3 SV=1;>tr|Q71V35|Q71V35_TOBAC ATP synthase subunit beta (Fragment) OS=Nicotiana tabacum GN=atpB PE=3 SV=1
0.392248352	0.857402597	non-significant	>tr|C3RXI5|C3RXI5_TOBAC Plastid transketolase OS=Nicotiana tabacum PE=2 SV=1
0.308822393	0.134619406	non-significant	>tr|Q9FSF6|Q9FSF6_TOBAC Ribosomal protein L11-like (Fragment) OS=Nicotiana tabacum GN=rbl11-like PE=2 SV=1
0.274839799	1.503352749	non-significant	>sp|P00876|RBL_TOBAC Ribulose bisphosphate carboxylase large chain OS=Nicotiana tabacum GN=rbcL PE=1 SV=2
0.265142639	1.024179991	non-significant	>tr|C5J0G6|C5J0G6_TOBAC Enolase OS=Nicotiana tabacum PE=2 SV=1
0.242072423	0.082419174	non-significant	>sp|P50218|IDHC_TOBAC Isocitrate dehydrogenase [NADP] OS=Nicotiana tabacum PE=2 SV=1
0.241951982	0.062064275	non-significant	>tr|O24662|O24662_TOBAC Aquaporin 1 OS=Nicotiana tabacum PE=2 SV=1;>tr|Q06BK4|Q06BK4_TOBAC Water channel protein OS=Nicotiana tabacum PE=2 SV=1;>tr|Q40595|Q40595_TOBAC Aquaporin OS=Nicotiana tabacum GN=NT2 PE=2 SV=1
0.219513456	0.086909026	non-significant	>tr|Q9ZP50|Q9ZP50_TOBAC ATP-dependent zinc metalloprotease FtsH OS=Nicotiana tabacum GN=Pftf PE=2 SV=1
0.218945901	0.729273787	non-significant	>sp|P69249|RBS_TOBAC Ribulose bisphosphate carboxylase small chain, chloroplastic OS=Nicotiana tabacum GN=RBCS PE=1 SV=1

LOG protein abundance ratio (GFP-VdAve1/ Ve1-HA	LOG average iBAQ	Significance	Fasta headers
0.205903411	0.127071838	non-significant	>tr|A1XEH7|A1XEH7_TOBAC CYP81B2v2 OS=Nicotiana tabacum PE=2 SV=1;>tr|A1XEH8|A1XEH8_TOBAC CYP81B2v1 OS=Nicotiana tabacum GN=TCP1 PE=2 SV=1
0.098224044	0.033576379	non-significant	>sp|Q03685|BIP5_TOBAC Luminal-binding protein 5 OS=Nicotiana tabacum GN=BIP5 PE=2 SV=1
0.075094223	0.099613839	non-significant	>tr|B5M4V6|B5M4V6_TOBAC Actin OS=Nicotiana tabacum PE=3 SV=1;>sp|Q05214|ACT1_TOBAC Actin OS=Nicotiana tabacum PE=3 SV=1;>sp|P93375|ACT7_TOBAC Actin-104 (Fragment) OS=Nicotiana tabacum PE=3 SV=1
0.068816145	0.253307623	non-significant	>sp|Q42961|PGKH_TOBAC Phosphoglycerate kinase, chloroplastic OS=Nicotiana tabacum PE=2 SV=1
0.047886411	0.016448904	non-significant	>sp|O80360|RK3_TOBAC 50S ribosomal protein L3, chloroplastic (Fragment) OS=Nicotiana tabacum GN=RPL3 PE=2 SV=1
-0.012617747	0.064252518	non-significant	>sp|P68158|EFTU_TOBAC Elongation factor Tu, chloroplastic OS=Nicotiana tabacum GN=TUFA PE=3 SV=1
-0.019102136	0.030768338	non-significant	>sp|P27141|CAHC_TOBAC Carbonic anhydrase, chloroplastic OS=Nicotiana tabacum PE=2 SV=1
-0.0468841	0.137598509	non-significant	>tr|Q1G0Z1|Q1G0Z1_TOBAC Putative spindle disassembly related protein CDC48 OS=Nicotiana tabacum PE=2 SV=1
-0.051922162	0.175682067	non-significant	>sp|Q9SMB4|PSBS_TOBAC Photosystem II 22 kDa protein, chloroplastic OS=Nicotiana tabacum GN=PSBS PE=2 SV=1
-0.069073121	0.678821451	non-significant	>sp|Q40565|RCA2_TOBAC Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic OS=Nicotiana tabacum GN=RCA PE=2 SV=1
-0.172990322	1.217637648	non-significant	>sp|P00823|ATPA_TOBAC ATP synthase subunit alpha, chloroplastic OS=Nicotiana tabacum GN=atpA PE=2 SV=2
-0.18727688	0.392323138	non-significant	>tr|Q84QE5|Q84QE5_TOBAC Ribulose bisphosphate carboxylase small chain OS=Nicotiana tabacum PE=2 SV=1
-0.193901539	0.091300126	contaminant	>sp|P04259|K2C6B_HUMAN Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5
-0.271076878	0.085185395	non-significant	>tr|Q8VXC9|Q8VXC9_TOBAC Alpha-tubulin OS=Nicotiana tabacum GN=tubA3 PE=2 SV=1;>tr|Q8VXD1|Q8VXD1_TOBAC Alpha-tubulin OS=Nicotiana tabacum GN=tubA1 PE=2 SV=1
-0.289611499	1.296102787	non-significant	>tr|Q5M9V4|Q5M9V4_TOBAC ATP synthase subunit alpha OS=Nicotiana tabacum GN=atp1 PE=3 SV=2
-0.370300174	0.158097998	non-significant	>tr|Q6TKR0|Q6TKR0_TOBAC Ribosomal protein L3A OS=Nicotiana tabacum GN=RPL3A PE=2 SV=1

LOG protein abundance ratio (GFP-VdAve1/ Ve1-HA	LOG average iBAQ	Significance	Fasta headers
-0.397721489	1.472635801	non-significant	>tr|Q0PWS5|Q0PWS5_TOBAC Chloroplast pigment-binding protein CP26 OS=Nicotiana tabacum GN=Lhcb5 PE=2 SV=1
-0.412149628	0.584973809	non-significant	>sp|P06413|PSBC_TOBAC Photosystem II CP43 chlorophyll apoprotein OS=Nicotiana tabacum GN=psbC PE=3 SV=1
-0.430381815	0.151631858	non-significant	>sp|P06357|RR3_TOBAC 30S ribosomal protein S3, chloroplastic OS=Nicotiana tabacum GN=rps3 PE=3 SV=1
-0.475889166	0.375400408	contaminant	>sp|P35527|K1C9_HUMAN Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3
-0.55174915	0.188523104	contaminant	>sp|P35908|K22E_HUMAN Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2
-0.55652829	0.529127994	contaminant	>sp|P04264|K2C1_HUMAN Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
-0.579170386	1.244279502	non-significant	>sp|P69686|PSBD_TOBAC Photosystem II D2 protein OS=Nicotiana tabacum GN=psbD PE=3 SV=1
-0.673024138	1.54125407	non-significant	>sp|Q03684|BIP4_TOBAC Luminal-binding protein 4 OS=Nicotiana tabacum GN=BIP4 PE=2 SV=1
-0.739972274	0.307824954	non-significant	>sp|P06247|CYB6_TOBAC Cytochrome b6 OS=Nicotiana tabacum GN=petB PE=2 SV=2
-0.816478491	0.439969777	non-significant	>sp|P09094|G3PC_TOBAC Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (Fragment) OS=Nicotiana tabacum GN=GAPC PE=2 SV=1
-1.128958821	2.039687591	contaminant	>sp|P00761|TRYP_PIG Trypsin OS=Sus scrofa PE=1 SV=1
-1.131083171	0.510349078	non-significant	>sp|Q03683|BIP3_TOBAC Luminal-binding protein 3 (Fragment) OS=Nicotiana tabacum GN=BIP3 PE=2 SV=1
-1.496753454	0.701922687	contaminant	>sp|P13645|K1C10_HUMAN Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
-1.737519026	1.230577265	non-significant	>tr|G9MD87|G9MD87_TOBAC Heat shock protein 90 OS=Nicotiana tabacum GN=NtHsp90er-2 PE=2 SV=1;>tr|G9MD86|G9MD86_TOBAC Heat shock protein 90 OS=Nicotiana tabacum GN=NtHsp90er-1 PE=2 SV=1
-2.83700943	3.891281809	significant	>VDJR2_10274_actin;>VDJR2_1519_actin;>VDJR2_10273_partial
-3.336211681	4.545364029	significant	>tr|Q3Y625|Q3Y625_TOBAC Sam3 OS=Nicotiana tabacum PE=4 SV=1;>tr|Q3Y636|Q3Y636_TOBAC Hav2 OS=Nicotiana tabacum PE=4 SV=1;>tr|Q3Y639|Q3Y639_TOBAC BY2 OS=Nicotiana tabacum PE=4 SV=1;>tr|Q94EW1|Q94EW1_TOBAC Nictaba OS=Nicotiana tabacum GN=NT1 PE=2 SV=1;>tr|Q3
-4.488051097	7.202087386	significant	>Ve1_Motelle;>Solyc09g005090.1.1 LRR receptor-like serine/threonine-protein kinase, RLP

Chapter 6

The tomato immune receptor Ve1 binds the *Verticillium dahliae* effector protein VdAve1 to recruit downstream signaling components

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Abstract

Receptor proteins with extracellular leucine-rich repeats (eLRR) are crucial components of the plant immune system. In tomato, the eLRR receptor protein Ve1 governs resistance against the vascular wilt pathogen *Verticillium dahliae* following recognition of the effector protein Ave1 (VdAve1). In contrast, no function could be ascribed to its homolog Ve2. Analyses of domain swap mutants between Ve1 and Ve2 demonstrated that the N-terminal eLRRs (the C1 domain) of Ve1 and Ve2 are functionally interchangeable. As the C1 domain has been shown to confer ligand specificity in other eLRR receptors, it has been hypothesized that it similarly mediates VdAve1 ligand perception. Here we show that both Ve1 and Ve2 interact with the VdAve1 effector. Deletion of four consecutive eLRR repeats that show the least amino acid difference between Ve1 and Ve2, does not result in the loss of binding to VdAve1. Thus, we hypothesize that this interaction is mediated by multiple clusters of eLRRs that are scattered along the C1 domain. Moreover, we confirm that Ve1 forms a ligand-induced receptor complex with the key regulatory eLRR receptor kinase BAK1 to initiate immune signaling, and discuss possible reasons for the lack of Ve2 functionality.

Introduction

Cell-surface receptors are essential components of plant surveillance systems detecting microbial invaders[1]. Recognition of appropriate ligands, which may be microbe-derived or result from microbe-mediated host manipulation, leads to the activation of immune responses to ward off potentially harmful microbes[2]. In turn, symbiotic microbes, including pathogens, endophytes as well as mutualists, secrete effector molecules that deregulate immune responses to successfully establish within their host[3,4].

In tomato, resistance to race 1 strains of the vascular wilt pathogen *Verticillium dahliae* is governed by the *Ve* locus, which encodes the two highly identical extracellular leucine-rich repeat receptor proteins (eLRR-RPs) Ve1 and Ve2[5,6]. Their extracellular domains consist of two eLRR regions (C1 and C3), which are separated by a non-eLRR island domain referred to as C2, and are anchored to the plasma membrane by a single-pass transmembrane domain. In contrast to eLRR-containing receptor kinases (eLRR-RKs), eLRR-RPs, including Ve1 and Ve2, carry cytoplasmic tails that lack obvious signaling domains[7]. Despite their identity, Ve1, but not Ve2, confers resistance to race 1 strains of *V. dahliae* in tomato and *Arabidopsis thaliana* (hereafter referred to as Arabidopsis)[8,9]. Additional homologs of Ve1 have been described to act as immune receptors in other plant species such as potato, wild eggplant, tobacco and hop, indicating that Ve1 is of ancient origin[10].

By using a comparative population genomics approach, the protein that is recognized by Ve1 was identified as *V. dahliae* Ave1 (VdAve1, for Avirulence on Ve1 tomato)[11]. During colonization of susceptible plant genotypes, VdAve1 greatly contributes to fungal virulence[11]. Transient *Agrobacterium tumefaciens*-mediated co-expression of Ve1 and VdAve1 in tobacco (*Nicotiana tabacum*) leaves resulted in a hypersensitive response (HR), a form of localized cell death indicative of ligand recognition by its corresponding plant immune receptor[11,12]. Similarly, transient potato virus X (PVX)-mediated expression of *VdAve1* induced HR in tomato carrying *Ve1*[11]. However, while the signaling cascade required for Ve1-mediated resistance is conserved in Arabidopsis, it does not involve an HR, suggesting that HR is not required for *Verticillium* wilt resistance[13].

Many eLRR-containing receptors recognize proteinaceous ligands and the specificity with which a ligand is recognized is usually determined by the eLRR domains[7,14-17]. Through domain swaps between Ve1 and Ve2, and targeted mutagenesis of Ve1, we have identified three separate clusters of Ve1 eLRR domains that are required for its function[18,19]. Two of these, namely eLRR1-8 and eLRR20-23, belong to the C1 domain and have been hypothesized to be involved in VdAve1 ligand binding[19]. In contrast, the third cluster that consists of eLRR32-37 was proposed to function in immune signaling activation[19].

Similar to other LRR-RPs, Ve1 and Ve2 constitutively associate with the eLRR-RK SUPPRESSOR OF BIR1-1 (SOBIR1) to form bimolecular equivalents of genuine eLRR-RKs[18,20-22]. Upon ligand perception, both eLRR-RKs and bimolecular eLRR-RP complexes initiate tightly

regulated cytoplasmic signal transduction cascades[1,23]. Recruitment of the regulatory BRI-1 ASSOCIATED RECEPTOR KINASE (BAK1; also known as SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3a (SERK3a)) and other members of the SERK family only occurs upon ligand perception and is required for eLRR-RP-mediated immunity, as was recently shown for Cf-4[24] and RLP23[25]. Likewise, SOBIR1 and BAK1 associate with, or are required for, immunity mediated by additional eLRR-RPs[22,26-30]. Interestingly, BAK1 also appears to have a negative regulatory role in eLRR-RP-mediated immunity as it was reported to attenuate xylanase-induced Eix2 immune activation in collaboration with the decoy receptor Eix1[31]. Genetic evidence also supports a regulatory role of BAK1 in Ve1-mediated resistance[6,9]. In this study, we further investigated the molecular mechanisms underlying Ve1 immune complex activation following VdAve1 ligand perception using a biochemical approach. In addition, based on previous findings and those presented here, we discuss the possibilities that explain non-functionality of Ve2 in *Verticillium* wilt resistance.

Results

Ve1 and Ve2 bind the effector protein VdAve1

Ve1 mediates resistance to *V. dahliae* race 1 strains upon perception of the VdAve1 effector protein[11]. To test whether Ve1 binds VdAve1, we first co-expressed GFP-tagged VdAve1 with untagged Ve1 in *Nicotiana tabacum* leaves to confirm its recognition (Suppl. Fig. 1a)[32]. As expected, co-expression of GFP-VdAve1 with Ve1 resulted in a hypersensitive response (HR) at 6 days post infiltration (dpi) (Suppl. Fig. 1b). This HR was comparable to the response observed when untagged Ave1 was co-expressed with Ve1[11]. Moreover, expression of GFP-Ave1 or Ave1 alone did not result in an HR (Suppl. Fig. 1b). To confirm the stability of GFP-Ave1 protein, we performed immunoprecipitation at 1, 2, 3 and 4 days following its transient expression in *N. benthamiana* and *N. tabacum* leaves. In both plant species the protein was stably produced at 1-2 dpi (Suppl. Fig. 1c). Due to the comparable ease of infiltration, we performed the following assays in *N. benthamiana*. Co-immunoprecipitation experiments were carried out following the transient production of affinity-tagged versions of Ve1, Ve2 and VdAve1 proteins at 2 dpi. The eLRR-RPs Cf9 from tomato and RLP30 from Arabidopsis served as negative controls. As expected, affinity-purification of Ve1-HA_3 resulted in co-purification of a protein of 39 kDa, which corresponds to the size of monomeric GFP-VdAve1 (Fig. 1a). A second, higher band was observed that could correspond to GFP-VdAve1 dimers, suggesting that effector dimers bind to Ve1. As expected based on the previously performed domain swap experiments, we similarly observed binding of monomeric and dimeric GFP-VdAve1 to Ve2-HA_3 (Fig. 1a). Importantly, GFP-VdAve1 did not co-purify with RLP30-HA_3 or Cf9-HA_3, confirming that the interaction of VdAve1 with Ve1 and Ve2 is specific (Fig. 1a).

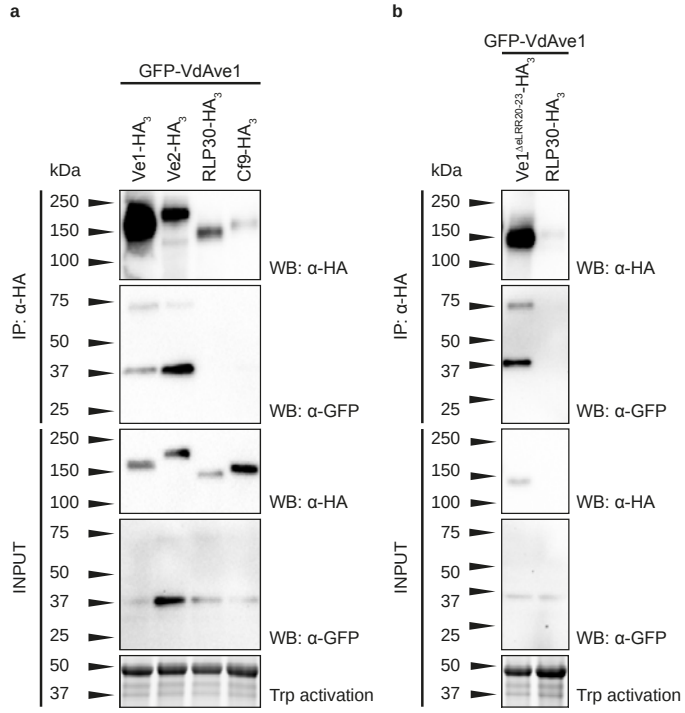

FIGURE 1 | Ve1 and Ve2 bind the fungal effector protein VdAve1. Affinity-tagged *Verticillium dahliae* Ave1 (VdAve1) was transiently co-expressed in *Nicotiana benthamiana* leaves with HA-tagged Ve1, Ve2, RLP30 and Cf9 (**a**) or Ve1$^{\Delta LRR20-23}$ and RLP30 (**b**). Leaf tissue was harvested at 2 dpi for total protein extraction (INPUT). Transiently expressed receptors were immunopurified with Anti-HA magnetic beads and co-purifying GFP-VdAve1 was visualized on Western blots (WB). Equal loading of protein samples onto TGX Stain-Free™ gels was determined by tryptophan (Trp) activation under UV light. The experiments were repeated twice.

eLRRs20-23 of Ve1 are not crucial for VdAve1 binding

Ve1 and Ve2 are share 84% amino acid identity, with the region between the eLRRs 19 and 23 being most highly conserved[5,18]. In a recent mutational screen, eLRR20 through eLRR23 were shown to be involved in VdAve1 recognition, since alanine substitutions of surface-exposed amino acid residues in any of the four eLRRs resulted in the loss of HR upon co-expression with VdAve1 in *N. tabacum*[19]. Because both Ve1 and Ve2 bind VdAve1, we generated a mutant construct of Ve1 lacking eLRR20-23 to test whether this highly conserved part of the extracellular region is required for effector binding. Interestingly, as with wild type Ve1, VdAve1 also co-purified with HA-tagged Ve1$^{\Delta eLRR20-23}$, whereas no binding to RLP30-HA$_3$ could be observed (Fig. 1b).

VdAve1 homologs interact with Ve1

VdAve1 homologs that occur in several phytopathogens are differentially perceived by tomato Ve1[32,33]. Thus, we hypothesized that the homologs recognized by Ve1 bind to

the receptor similar to VdAve1. We tested the interaction between the receptors and the VdAve1 homologs of *V. nubilum* (VnAve1), *Fusarium oxysporum* (FoAve1), *Cercospora beticola* (CbAve1), *Colletotrichum higginsianum* (ChAve1), and *Xanthomonas citri* subsp. *citri* (XacPNP) following their transient production in *N. benthamiana* leaf tissue. Like VdAve1, all GFP-tagged monomers and multimers of the VdAve1 homologs co-purified with Ve1-HA_3 but not with RLP30-HA_3 (Fig. 2). Intriguingly, we also observed co-purification of VnAve1 and FoAve1 in Ve2-HA_3 affinity purifications whereas CbAve1, ChAve1 and XacPNP did not co-purify with Ve2 (Fig. 2).

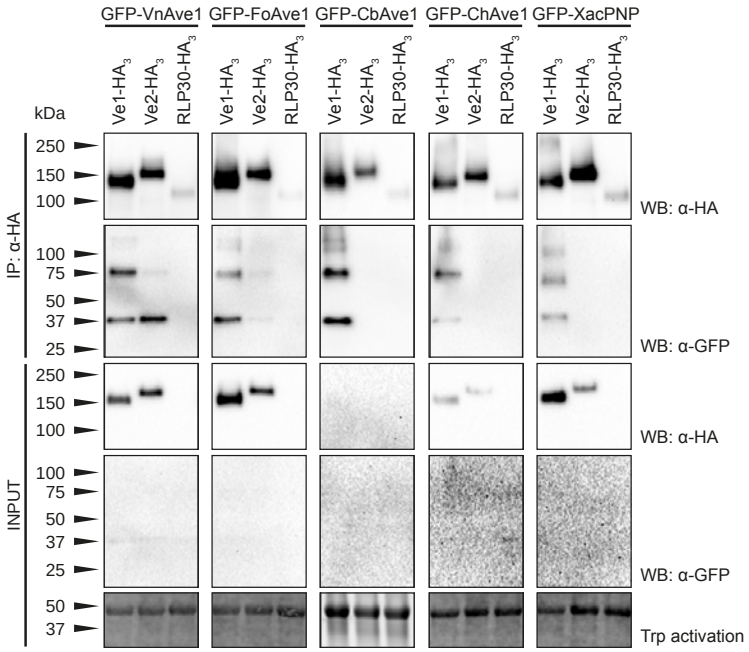

FIGURE 2 | Ve1 binds microbial Ave1 homologs. GFP-tagged VdAve1 homologs from *Verticillium nubilum* (Vn), *Fusarium oxysporum* (Fo), *Cercospora beticola* (Cb), *Colletotrichum higginsianum* (Ch), and *Xanthomonas citri* subsp. *citri* (Xac) were transiently co-expressed with Ve1-HA_3 and Ve2-HA_3 tomato receptor proteins in *Nicotiana benthamiana* leaves. Co-expression with RLP30-HA_3 was used as negative control. Receptor proteins were purified with Anti-HA magnetic beads from total leaf extracts (INPUT) at 2 dpi. Co-purifying effectors were visualized on Western blots (WB) and total proteins were visualized by tryptophan (Trp) activation under UV light to test equal loading. The experiment was repeated twice.

Ve1 recruits BAK1 upon VdAve1 ligand perception

Ve1 constitutively interacts with the eLRR-RK SOBIR1[18] and previous genetic analysis revealed that Ve1-mediated resistance depends on *BAK1*[9]. Thus, we tested whether the Ve1-SOBIR1 complex recruits BAK1 upon ligand perception. To this end, Ve1-eGFP was transiently expressed together with *Sl*SOBIR1-HA and *Sl*BAK1-cMyc in *N. benthamiana* leaves. Two days later, leaves were either infiltrated with GST-tagged VdAve1 or GST alone. Preliminary results show that *Sl*BAK1-cMyc specifically co-purifies with Ve1-eGFP

after elicitation with GST-VdAve1 but not GST (Fig. 3). This demonstrates that Ve1 forms a ligand-dependent receptor complex with BAK1, similar to Cf-4 and RLP30[24,25]. In contrast, *Sl*SOBIR1-HA was detected upon immunopurification of Ve1-eGFP following infiltration with both GST and GST-VdAve1 (Fig. 3), confirming the ligand-independent interaction between SOBIR1 and LRR-RPs[18,22].

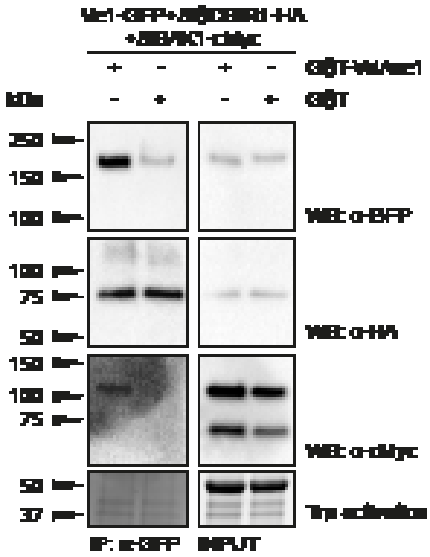

FIGURE 3 | Ve1 recruits BAK1 in a ligand-dependent manner. Ve1-eGFP, *Sl*SOBIR1-HA and *Sl*BAK1-cMyc were transiently co-expressed in *Nicotiana benthamiana* leaves. At 2 dpi, 10 µM purified GST-VdAve1 or GST were infiltrated into transformed leaves. Total protein was extracted after 30 min of incubation followed by α-GFP affinity purification. Co-purifying proteins were visualized on Western blots (WB). Equal loading of protein samples onto TGX Stain-Free™ gels was determined by tryptophan (Trp) activation under UV light. The experiment was done once.

Discussion

Plant eLRR-carrying surface-localized receptor proteins play crucial roles in development and immunity. While the ligands of many eLRR receptors have been identified, evidence for the physical receptor-ligand interaction is often still lacking. Here we show that the eLRR-RP Ve1 and its non-functional homolog Ve2 from tomato bind the *V. dahliae* effector protein VdAve1 (Fig. 1a). This confirms the earlier hypothesis that Ve2, like Ve1, would be able to interact with VdAve1 since the C1 domains of Ve1 and Ve2 are functionally interchangeable[18]. Similarly, functional dissection of the tomato Cf receptors Cf-4 and Cf-9, eLRR-RPs that provide resistance to the leaf mold fungus *Cladosporium fulvum*, by domain swap analyses showed that their eLRRs13-16 and eLRRs10-16 contribute to ligand specificity, respectively[17,34]. More recently, expression of chimeric mutants of tomato Eix2 carrying the eLRR region of the eLRR-RP ReMAX resulted in responsiveness to the ReMAX ligand eMAX in *N. benthamiana*, which lacks an endogenous perception system for this ligand[28]. In addition, sequential domain swaps between the eLRR-RKs EFR and FLS2 highlighted the importance of the EFR eLRRs for the perception of its ligand EF-Tu[35]. These findings demonstrate that the eLRR regions of eLRR-carrying receptors determine ligand specificity.

While some receptors were shown to bind their ligands directly[25,35-37], others perceive their ligands indirectly by monitoring plant molecules that are targeted by effector proteins. This is the case for tomato Cf-2, which does not directly interact with the *C.fulvum* effector Avr2 but binds tomato papain-like cysteine proteases that are inhibited by Avr2[38-40]. Similarly, Cf-9 does not directly bind *C. fulvum* Avr9 but requires a high-affinity binding site[41,42]. In addition, many intracellular nucleotide-binding domain leucine-rich repeat receptors (NLRs) recognize pathogen effectors indirectly[43]. This is the case for the Arabidopsis NB-LRR receptors RPM1 and RPS2, both of which recognize modifications of the RIN4 protein by the *Pseudomonas syringae* effectors AvrB or AvrRPM1 and AvrRpt2, respectively[44-46]. Whether or not the interaction of Ve1 and Ve2 with VdAve1 is direct or indirect cannot be concluded from our experiments, and still needs to be confirmed. After all, the plant species that were used for the protein interaction studies may share the effector target.

Phylogenetic analysis of VdAve1 resulted in the identification of homologs in the fungal pathogens *F. oxysporum*, *C. higginsianum*, and *C. beticola*, the saprotroph *V. nubilum*, as well as in the biotrophic bacterial pathogen *Xanthomonas citri* subsp. *citri*[11]. Strikingly, however, most homologs were found in plants, with the most closely related homologs present in tomato (*Solanum lycopersicum*; SlAve1) and grape (*Vitis vinifera*; VvPNP)[11]. Co-expression of these homologs with *Ve1* in *N. tabacum* was previously shown to result in distinct patterns of HR, corresponding to their increasing divergence from VdAve1[32]. Interestingly, however, despite the differences in recognition by Ve1, all microbial homologs bind the tomato receptor, similar to VdAve1 (Fig. 2). This suggests that the binding properties are conserved between VdAve1 and its homologs but that the physical receptor-ligand interaction is not sufficient for the activation of Ve1-mediated immunity. Potentially, this could be attributed to differential strengths of the interactions, which may be supported by the finding that VnAve1 and FoAve1 co-purify with Ve2-HA_3, whereas CbAve1, ChAve1 and XacPNP do not.

The region composed of eLRR20-23 displays very few amino acid differences between Ve1 and Ve2, and has previously been shown to be required for VdAve1 recognition and Ve1-mediated resistance to *V. dahliae*[19]. Since both receptors interact with the VdAve1 ligand, we tested whether eLRR20-23 are required for VdAve1 binding. Deletion of this subdomain in Ve1 did not result in loss of effector binding (Fig. 1b), indicating that the region between eLRR20-23 is not sufficient for the interaction with VdAve1. In addition to eLRR20-23, a second cluster of eLRRs (eLRR1-8) was shown to be required for Ve1 functionality[19]. Thus, it is conceivable that the physical interaction between Ve1 (and Ve2) and VdAve1 may be mediated by (a surface formed between) the two separate clusters of eLRRs. This is in contrast to the architecture of the ligand binding sites of other eLRR-containing receptors. For example, crystallographic analyses demonstrated that the eLRR-RK BRASSINOSTEROID INSENSITIVE 1 (BRI1) binds its brassinolide ligand via a hydrophibic groove formed by the island domain and the surrounding eLRRs20-25[16,47]. Similarly, as

elegantly shown by photoaffinity labeling, phytosulfokine (PSK) binding to the PSK receptor PSKR1 is mediated by the island domain[48,49]. Instead, the receptor kinases BAM1 and FLS2, which lack a non-LRR island domain, directly interact with the CLE9 and flg22 peptides, respectively, through a contiguous stretch of eLRRs[50,51].

Due to the absence of cytoplasmic signaling domains, eLRR-RPs rely on the association with receptor kinases that function as adapters and co-receptors to activate appropriate responses upon ligand perception. Recent work has shown that SOBIR1 acts as a common adaptor to eLRR-RPs to form constitutive bimolecular receptor complexes[20,21], including the immune receptors Cf-4, Cf-9 and Eix1 from tomato, RLP23 from Arabidopsis, as well as the *Leptosphaeria maculans* receptor 3 (LepR3) from oilseed rape[22,25,26,30]. In addition, the Arabidopsis eLRR-RPs ReMAX, RLP30 and RLP42 require SOBIR1 for their function in immune signaling[27,28,52]. Also Ve1 has been shown to associate with SOBIR1 in a ligand-independent manner[18]. Due to the high sequence conservation of the juxtamembrane C3 domain between various eLRR-RPs and because this region is important for Ve1 function, it has been hypothesized that the C3 domain is involved in the interaction with SOBIR1[19]. However, this domain is not functional in Ve2[18]. Nonetheless, like Ve1, Ve2 forms a ligand-independent complex with SOBIR1. This suggests that the amino acid residues (or their properties) required for SOBIR1 binding are conserved between Ve1 and the non-functional Ve2 receptor and that the association with SOBIR1 may not be a determining factor of Ve1-mediated immunity[18]. Moreover, it does not explain the lack of Ve2 functionality in resistance to *V. dahliae*.

Upon ligand perception, eLRR-containing receptor molecules recruit additional receptor complex components to activate intracellular signaling cascades. BAK1 acts as a common co-receptor to both eLRR-RPs and eLRR-RKs to regulate developmental as well as immunological processes[53]. We show here that the Ve1-SOBIR1 complex recruits BAK1 in a ligand-dependent manner (Fig. 3), as has previously been demonstrated for FLS2, Cf-4, and RLP23[24,25,54]. This is in accordance with the previous finding that BAK1 silencing impairs Ve1-mediated immunity in tomato[6]. Considering that ligand-dependent complex formation with BAK1 appears to be a common feature of eLRR receptor-mediated immunity, and since Ve2 interacts with the SOBIR1 receptor adapter, it is tempting to speculate that Ve2 does not activate immune signaling upon VdAve1 binding because it fails to recruit BAK1. This hypothesis is supported by the finding that the C-terminus of Ve2, which is 91 amino acids longer than that of Ve1, interferes with Ve1 function in domain swap mutants[18]. However, while Ve1 and Ve2 appear to have the same subcellular localization in tobacco cells, we cannot exclude the possibility that the PEST-like sequence in the Ve2 C-terminus, which is commonly found in proteins with short half-lives[5], contributes to Ve2 degradation and, thus, renders it non-functional. Future studies, especially the structural analysis of the ligand-induced Ve1 receptor complex, will help to address this question.

Materials

Plant material

Nicotiana benthamiana plants were grown in the greenhouse at 21°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity. When natural light intensities dropped below 150 W/m^2, supplemental light was provided at 100 W/m^2. For agroinfiltration, plants were transferred to climate chambers set to 21°C/19°C during 12/12 hours day/night periods, respectively, with 70% relative humidity.

Constructs for *Agrobacterium tumefaciens*-mediated transient expression

Receptor expression constructs pSol2095:Ve1-eGFP, pB7K40:Ve1-HA$_3$, pB7K40:Ve2-HA$_3$, pBIN-KS-35S:RLP30-HA$_3$, pGWB20:SlBAK1-cMyc$_{10}$, and pBIN-KS-35S:SlSOBIR1-eGFP as well as effector expression constructs pSol2092:GFP-VdAve1, pSol2092:GFP-VnAve1, pSol2092:GFP-FoAve1, pSol2092:GFP-CbAve1, pSol2092:GFP-ChAve1, pSol2092:GFP-XacPNP and pSol2092:SP-GFP were previously described[12,18,22,24,32]. C-terminal VdAve1 deletion mutants were cloned from pEX-A2:SP-GFP-VdAve1 synthesized by Eurofins (Ebersberg, Germany) using the primers shown in Supplementary Table 1. The PCR-amplified fragments were cloned into pDONR207 by using the Gateway® BP Clonase® II Enzyme Mix (Invitrogen, California, USA) to generate the entry vectors pDONR207::VdAve1$^{\Delta 126\text{-}134}$ and pDONR207::VdAve1$^{\Delta 117\text{-}134}$. The constructs were verified by DNA sequencing (Eurofins Genomics, Ebersberg, Germany) and transferred into the Gateway-compatible destination vector pSol2092[12] by using Gateway® LR Clonase® II Enzyme Mix (Invitrogen, California, USA).

A. tumefaciens-mediated transient expression (agroinfiltration)

Overnight cultures of *A. tumefaciens* strain GV3101 carrying the respective expression constructs were centrifuged and pellets were re-suspended in MMA medium (2% sucrose, 0.5% Murashige & Skoog salts (Duchefa Biochemie, Haarlem, The Netherlands), 10 mM MES, 200 µM acetosyringone, pH 5.6 with NaOH). Bacterial suspensions were mixed at a 1:1 ratio for co-expression of effector with receptor genes. For co-expression of *Ve1-HA*/*Ve2-HA*, *SlSOBIR1-GFP*, and *BAK1-cMyc*, bacterial suspensions were mixed at a 1:1:1 ratio. Mixtures were infiltrated into leaves of 4 to 6 week-old *N. benthamiana* plants. After 24-48 hours of co-expression, infiltrated leaves were harvested and snap-frozen in liquid N_2.

Co-immunopurifications and immunoblotting

Ground leaf material was used for total protein extraction with 2 mL extraction buffer (150 mM Tris, 150 mM NaCl, 10% glycerol, 10 mM EDTA, 10 mM DTT, 0.5% PVPP, protease inhibitor cocktail (Roche, Mannheim, Germany), 1% IGEPAL CA-630 (NP40), pH 7.5) per gram

of leaf material. Five mL total protein extracts were incubated with 20 µL Pierce™ Anti-HA magnetic beads (Thermo Scientific, Eindhoven, The Netherlands) or GFP-Trap®_A beads (Chromotek, Planegg-Martinsried, Germany) shaking for 1 hour at 4°C. Beads were washed three times with 1x TBS-T(20). Proteins were eluted from beads in 30 µL 2x Laemmli sample buffer (120 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.02% (w/v) bromophenol blue) by boiling at 95°C for 5 min. Proteins were separated on 4-20% gradient Mini-PROTEAN® TGX Stain-Free™ Precast Protein Gels (BioRad, Veenendaal, The Netherlands) in 1x Tris/glycine/SDS running buffer (BioRad, Veenendaal, The Netherlands) at 150 V for 1 hour. Protein bands were transferred onto Immun-Blot® PVDF membranes (BioRad, Veenendaal, The Netherlands) in blotting buffer (247.7 mM Tris, 1.92 M glycine, 10% ethanol) using the Trans-Blot® Turbo™ Blotting System (BioRad, Veenendaal, The Netherlands) for 30 min at 25 V. Membranes were blocked in 4% bovine serum albumin (BSA) in 1x TBS-T(20). To detect GFP-tagged proteins, blots were incubated with 1:5,000 diluted αGFP-HRP (Anti-GFP-HRP, 130-091-833, MACS antibodies). HA fusion proteins were detected with 1:2,500 diluted αHA-HRP (3F10; Roche, Mannheim, Germany) and cMyc-tagged proteins were detected with 1:5,000 α-cMyc-HRP (Thermo Scientific, Eindhoven, The Netherlands). Bands were visualized using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, Eindhoven, The Netherlands).

Recombinant protein

GST-tagged Ave1 was heterologously produced in insect cells and purified by GenScript (Piscataway, NJ, USA). GST was purchased from Genscript (Piscataway, NJ, USA).

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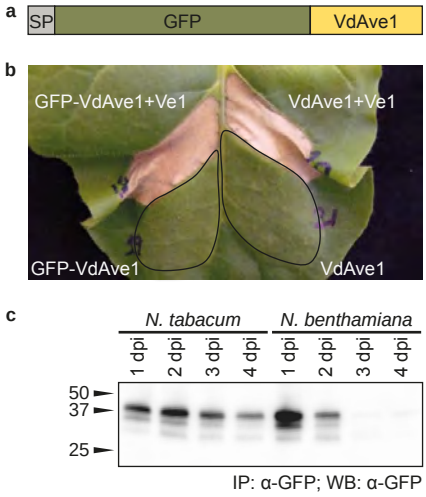
Supplementary data



SUPPLEMENTARY FIGURE 1 | GFP-tagged VdAve1 is expressed and recognized by Ve1. a, Graphic representation of the *GFP-VdAve1* construct used for *A. tumefaciens*-mediated transient expression in *N. tabacum* and *N. benthamiana*. **b**, Hypersensitive response (HR) following co-expression of GFP-tagged or untagged VdAve1 with Ve1 in *N. tabacum* at 6 dpi. Expression of GFP-VdAve1 or Ave1 alone did not result in HR. **c**, Western blot (WB) showing GFP-VdAve1 stability at 1, 2, 3, and 4 days following *A. tumefaciens*-mediated transient expression in *N. tabacum* and *N. benthamiana*. GFP-VdAve1 was immunopurified with α-GFP Trap beads from total protein extracts.

Chapter 7

General discussion

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This thesis provides a detailed insight into the mechanisms underlying chitin-triggered immunity in tomato, and the strategies that evolved in the two fungal pathogens *Cladosporium fulvum* and *Verticillium dahliae* to avoid immune activation. The presented work represents a significant advancement in our understanding of how fungal microbes manipulate their host's immune system by actively targeting its constituents, including chitinases and chitin receptors. In the following sections, these findings are placed into the broader context of immune recognition of cell wall-derived molecules and the evolution of fungi as well as oomycetes, collectively referred to as filamentous microbes, toward its suppression.

Introduction

The first intimate contact between host plants and filamentous microbes is often established in the apoplast; the extracellular spaces of plant tissues. While the apoplast provides nutrients and shelter to microbial inhabitants, it also represents a hostile environment with host-derived toxins and hydrolytic enzymes such as chitinases and glucanases that affect pathogen cell wall integrity (Fig. 1a). Moreover, the released cell wall fragments may serve as invasion patterns (IPs) and activate host immune receptors[1-3]. Consequently, microbial mechanisms evolved to circumvent cell wall-triggered immune responses and support host colonization.

Recognition of glycan oligomers is mediated by closely related receptors

Chitin is an important structural building block of fungal cell walls and a well-known elicitor of immune responses in plants[1]. Chitin perception by surface-localized lysin motif (LysM)-containing receptors and subsequent immune activation are well understood[1,4]. In rice, chitin perception is mediated by the LysM receptor OsCEBiP, which lacks an intracellular signaling domain[5]. Two OsCEBiP molecules simultaneously bind a single chitin heptamer-octamer, resulting in the recruitment of the LysM receptor kinase OsCERK1 (Fig. 1b)[6]. OsCERK1 is then phosphorylated and initiates immune signaling[6,7]. Additionally, OsCERK1 forms multimeric complexes with LysM receptors implicated in peptidoglycan perception[8,9]. Thus, OsCERK1 functions as signal transduction adaptor to multiple LysM immune receptors in response to the recognition of various microbial glycans.

Despite considerable efforts, the proposed composition of the chitin receptor complex in Arabidopsis remains controversial. Similar to rice, the Arabidopsis CERK1 homolog plays a central role in chitin-triggered immune signaling[10]. However, unlike OsCERK1, AtCERK1 was reported to directly bind long chain chitin oligomers, albeit with relatively

low affinity[11-13]. Recently, the LysM receptor AtLYK5, which lacks an active kinase domain, was proposed as primary chitin receptor instead of AtCERK1 based on its higher affinity for chitin[14]. Similar to rice, AtLYK5 forms a chitin-induced heteromeric complex with AtCERK1, triggering AtCERK1 phosphorylation and immune signaling activation. Since AtCERK1 functions in additional processes including peptidoglycan recognition and bacterial immunity[15], AtCERK1 may be a receptor adaptor, equivalent to *Os*CERK1. As in Arabidopsis, chitin recognition in tomato is mediated by a LysM receptor, called SlLYK4, which lacks an active intracellular kinase domain (Chapter 4). Thus, it is likely that SlLYK4 recruits a second receptor component, such as CERK1 in Arabidopsis and rice, to initiate immune signaling upon chitin perception.

In contrast to chitin, little is known about glucan recognition in plants. Due to their abundance in fungal and oomycete cell walls, glucan oligosaccharides likely evoke plant immune responses (Fig. 1b)[2]. The only known ß-glucan receptor, soybean GBP, does not contain LysM domains but specifically binds a hepta-ß-glucoside via a glucan-binding site[16]. As GBP lacks an intracellular signaling domain, it is probably part of a multimeric receptor complex at the cell surface, analogous to chitin perception complexes.

Interestingly, in addition to immunity, LysM receptors enhance the efficiency of the establishment of mutualistic symbioses triggered by short chain chitin fragments and chitin-related lipochitooligosaccharides, called Myc factors[17,18]. Some LysM receptors play a dual role in the perception of Myc factors and closely-related bacterial Nod factors that trigger root nodule symbiosis[17]. Moreover, *Os*CERK1 has recently been shown to function both in immunity and mutualism[19,20].

Biochemical analyses showed that several LysM receptors are glycosylated with high-mannose-type glycans or complex-type *N*-glycans[5,21]. However, to date no functional role has been ascribed to LysM receptor *N*-glycosylation[22]. Thus, the importance of such posttranscriptional modifications for ligand binding and receptor function still needs to be determined.

Collectively, the findings discussed above highlight the central role of LysM receptors in the recognition of chitin-derived microbial ligands. Future work will have to determine whether the perception of other cell wall glycans is similarly mediated by LysM receptors or by different classes of receptor proteins.

Microbial strategies to evade glycan-triggered immunity

The recognition of microbe-derived glycan molecules activates immune responses that hamper host colonization. In order to overcome or bypass such responses, filamentous microbes employ different strategies, comprising cell wall modifications and the secretion

of glycan-binding effector proteins. These effectors either sequester glycan elicitors and prevent their recognition or shield the cell wall from hydrolysis. Additionally, filamentous microbes secrete effectors that inhibit hydrolytic enzyme activity and proteases that cleave hydrolytic enzymes.

Cell wall modifications

The cell wall of filamentous microbes is vital for their growth, morphogenesis and survival. Despite variations between species, chitin and ß-glucan are the most abundant cell wall components. Their recognition by host surface-localized receptors activates detrimental immune responses. In contrast, deacetylated chitin (chitosan) and α-glucans are much weaker inducers of defense responses in most plants[2]. Moreover, chitosan is a poor substrate for plant chitinases[23]. Therefore, conversion of chitin to chitosan may protect hyphae against cell wall hydrolysis, and limit the release of plant immune signaling elicitors (Fig. 1d)[1]. Affinity labeling of invasive hyphae of particular fungal pathogens with a chitosan-specific antibody but not a chitin-binding probe suggested that cell wall chitin was replaced by chitosan[24]. Moreover, genome analyses of the ectomycorrhizal fungus *Laccaria bicolor* and the phytopathogen *Melampsora larici-populina* revealed an expanded repertoire of chitin deacetylases[25,26]. Thus, chitin deacetylation may represent a widespread mechanism of fungal cell wall protection and plant immune signaling evasion (Fig. 1d).

Rather than deacetylating chitin, some pathogens accumulate α-1,3-glucan on the surface of infectious hyphae (Fig. 1c)[27]. Since mutants with reduced levels of α-1,3-glucan are more susceptible to chitinases and display reduced virulence, α-1,3-glucan may protect fungal cell walls by masking chitin and ß-glucans (Fig. 1c)[27]. An additional strategy for evading ß-glucan-triggered immunity is the depletion of this polymer at the cell wall of biotrophic hyphae. While β-1,3-glucan synthesis is required for cell wall rigidity in appressoria and fast-growing necrotrophic hyphae of the maize pathogen *Colletotrichum graminicola*, during biotrophic development its synthesis is rigorously downregulated[28].

In conclusion, by converting, depleting or disguising highly immunoactive cell wall components, filamentous microbes can prevent cell wall hydrolysis and the release of host immune signaling elicitors.

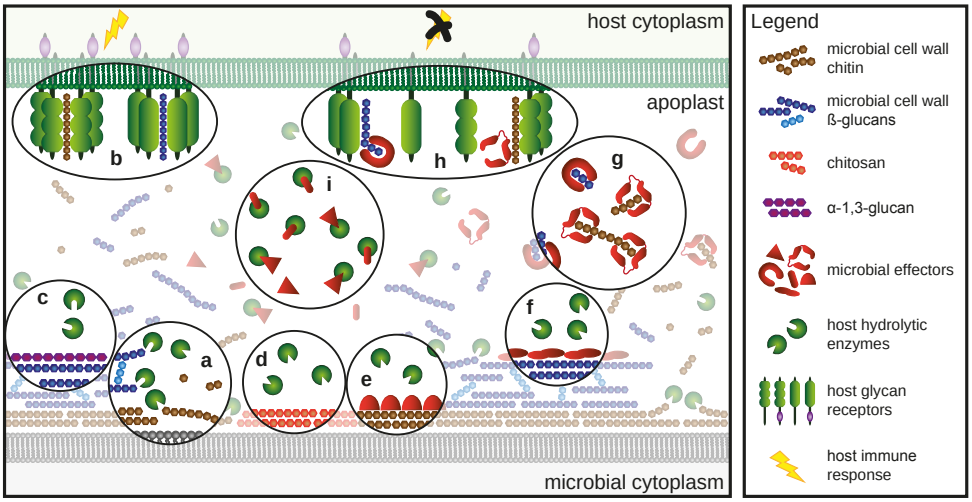

FIGURE 1 | Illustration of potential mechanisms underlying the activation of glycan-triggered host immune responses and microbial strategies to evade glycan recognition. (a) Microbial cell wall glycans are targeted by host-derived hydrolytic enzymes, including glucanases and chitinases, resulting in the release of glycan fragments. (b) Recognition of chitin oligomers by plasma membrane-localized host receptors results in the assembly of oligomeric receptor complexes, leading to the activation of immune responses. Similarly, recognition of ß-glucan fragments is anticipated to occur at the host cell surface. (c) Microbial cell wall remodeling may reduce the access of hydrolytic enzymes to particular cell wall glycans to prevent their hydrolysis, such as accumulation of α-1,3-glucan at the surface. (d) Certain fungal species convert chitin to chitosan, which is less immunogenic and a poor substrate to chitinases. Microbial secretion of glycan-binding effector proteins may shield cell wall chitin (e) or glucans (f) from hydrolysis, or sequester released glycan fragments to prevent their recognition (g). (h) Particular effectors may prevent the assembly or activation of host receptor complexes by inhibiting ligand-induced receptor dimerization. (i) Finally, microbes may secrete effectors that directly inhibit host hydrolytic enzymes.

Sequestration of cell wall-derived glycan fragments

Chitin-binding LysM effector proteins are versatile suppressors of chitin-triggered immunity (Fig. 1e,g,h). The *Cladosporium fulvum* LysM effector Ecp6 is one of the most abundant apoplastic proteins during tomato infection that binds chitin with high specificity and perturbs chitin-triggered immune responses[29,30]. Crystallization of Ecp6 demonstrated that two of its three LysM domains form a composite binding site with ultrahigh (pM) chitin affinity, enabling Ecp6 to outcompete host immune receptors for chitin binding[31]. Interestingly, the singular LysM2 domain of Ecp6, which is not involved in intramolecular chitin binding, retains the capacity to suppress chitin-triggered immune responses. Thus, it has been hypothesized that LysM2 of Ecp6 may interfere with chitin-induced host receptor dimerization to prevent activation of immune signaling (Fig. 1h)[31,32]. First evidence in support of this hypothesis results from functional analyses in Arabidopsis that suggest that Ecp6 does not prevent AtCERK1 phosphorylation but

affects the internalization of AtLYK5 in a ligand-dependent manner (Chapter 3). With the identification of the tomato chitin receptor, the biological relevance of these findings can now be confirmed in the *C. fulvum* host.

Like Ecp6, *Magnaporthe oryzae* Slp1, *Zymoseptoria tritici* Mg3LysM, and *Colletotrichum higginsianum* ChELP1 and ChELP2 deregulate chitin-triggered immunity[33-35]. Therefore, the ability to interfere with chitin recognition and immunity is conserved between fungal pathogens of different genera. Interestingly, Slp1 stability and activity depend on *N*-glycosylation by the α-1,3-mannosyltransferase AGL3[36]. Similarly, ChELP1 and ChELP2 appear to be *N*-glycosylated[35] suggesting that the addition of glycans may be an important feature of LysM effector function.

Importantly, the root endophyte *Serendipita indica* has recently been shown to secrete a fungal-specific lectin, FGB1, to compete with Arabidopsis and barley β-glucan receptors to suppress β-glucan-induced defenses in root and leaf tissues[37]. This suggests that, in addition to chitin, recognition of ß-glucans sparked the evolution of ß-glucan-binding effectors to interfere with ß-glucan-triggered immune responses (Fig. 1g,h).

Cell wall masking and inhibition of hydrolytic enzyme activity

In addition to chitooligosaccharide sequestration, *Z. tritici* Mg3LysM protects fungal hyphae from degradation by plant chitinases[33]. *Z. tritici* secretes a second LysM effector comprising a single LysM domain, called Mg1LysM, which displays protective activity as well. Although the molecular mechanism underlying hyphal protection is unknown, these LysM effectors may form chitin-dependent oligomeric chains as protective layers around invasive hyphae (Fig. 1e)[1]. This was previously demonstrated for the *C. fulvum* effector Avr4 and a *Pseudocercospora fijiensis* orthologue that bind polymeric chitin via an invertebrate chitin-binding site to protect hyphae against hydrolysis[38,39]. Similarly, effectors to mask cell wall ß-glucan may exist (Fig. 1f).

Alternatively, Mg3LysM and Mg1LysM may protect hyphae through direct inhibition of chitinase activity (Fig. 1i). *In silico* analyses of structural models demonstrated that particular active site residues of plant class I chitinases are subject to positive selection pressure, likely imposed by microbial chitinase inhibitors[40]. Intriguingly, the effector VdAve1 of the vascular pathogen *V. dahliae*, which belongs to a group of ubiquitous plant natriuretic peptide (PNP)-containing proteins, is able to inhibit a class I chitinase of tomato (Chapter 5). This function is not displayed by its closest homolog from the saprotroph *V. nubilum*, and explains the earlier observation that core LysM effectors of *V. dahliae* are not expressed *in planta*, and thus do not contribute to virulence through suppression of chitin-triggered immunity[41]. Additionally, fungalysin metalloproteases can cleave class IV chitinases[42]. In *Fusarium oxysporium* f.sp. *lycopersici* a serine protease and metalloprotease contribute to virulence and proteolytically cleave extracellular tomato chitinases[43]. Similar

activities occur in other tomato pathogens, suggesting that targeting of chitinases is a general strategy to protect cell walls[43].

Further evidence for the direct inhibition of plant hydrolytic enzymes is provided by the glucanase inhibitor protein 1 (GIP1) of *Phytophthora sojae*, which directly inhibits a soybean endo-ß-1,3-glucanase (EGaseA)[44]. Similar to class I chitinases, Glycine EGaseA enzymes are under positive selection pressure and display high variability at residues in close proximity to the GIP1 inhibitor binding site[45]. Moreover, the cell death-inducing mycotoxin Fumonisin B1 of *Fusarium verticillioides* also inhibits basic ß-1,3-glucanases in maize embryos[46]. Thus, filamentous microbes evolved various protective mechanisms against host hydrolytic enzymes, including chitinases and glucanases (Fig. 1i).

Evasion strategies of animal-infecting fungi

In mammals, fungal cell walls are the main source of elicitors whose recognition leads to the activation of immune responses including phagocytosis and fungal clearance[47]. Recognition of chitin, ß-glucans and mannosylated proteins is mediated by various receptor molecules, including C-type lectin and Toll-like receptors[48]. Consequently, also animal-infecting fungi evolved to mask and subvert host recognition. For example, *Histoplasma capsulatum* and *Aspergillus fumigatus* disguise immunoactive cell wall ß-glucans by depositing surface α-glucans that have little to no immunostimulatory activity[49,50]. In contrast, *A. fumigatus* conidia are coated with hyprophobins that are covalently bound to cell wall polysaccharides to prevent immune recognition[51].

The lack of an identified chitosan receptor and conflicting reports on its immunological activity suggest that chitosan is not a major elicitor of immunity in animals[52]. Thus, chitin deacetylation to chitosan may shield invasive hyphae from recognition and hydrolytic cleavage. Supporting evidence is provided by a recent genomic survey on the opportunistic mammalian pathogen *Rhizopus oryzae*, which unveiled an expanded repertoire of chitin deacetylases[53]. Thus, in addition to cell wall masking, cell wall modifications may play an important role in the evasion of glycan-triggered immunity by animal pathogens.

Despite their ubiquitous occurrence, animal pathogen LysM effectors have thus far not been found to suppress host immunity. It has been hypothesized that infection of mammalian hosts does not rely on effector activity due to their low degree of host adaptation and absence of host specificity[54]. This is supported by the absence of LysM effector genes in various mammalian commensals[55].

Conclusions

The involvement of glucans, chitin, chitooligosaccharides and their derivatives in plant-microbe communication exemplifies how cell-surface exposed and free glycans regulate complex processes during disease manifestation or establishment of mutualistic symbiosis in plants. As many glycans have the potential to become immunogenic in host organisms, filamentous microbes rely on diverse strategies to prevent the activation of host immune responses. Both animal and plant-infecting filamentous microbes modify and mask immunoactive components of their cell walls. Moreover, the role of fungal pathogen-derived LysM effectors in the suppression of plant chitin-triggered immunity has been intensely studied. Recent evidence suggests that, depending on their cell wall composition, fungal and oomycete symbionts may employ a suite of effector proteins to protect, sequester, and mask chitin and glucan cell wall components alike. Moreover, some effectors inhibit hydrolytic enzyme activity. Whether this inhibition is based on effector binding to the enzyme-glycan complex or the active site itself remains to be determined. Therefore, the basic requirement to circumvent glycan-triggered immunity is a driving force in the convergent evolution of all filamentous microbes toward its suppression.

The use of oligosaccharides as signaling molecules in plants and the adoption of cell-surface glycan receptors illustrate how glycans have been adapted to serve new functions during evolution. Thus, better understanding of the factors that contribute to maintenance of harmful or beneficial microbial glycan structures is required as a basis for fundamental breakthroughs in knowledge of disease development and growth promotion in plants. The availability of a large number of filamentous microbe and plant genomes to identify putative microbial glycan-binding proteins and respective plant receptors, as well as recent advances in glycan research in animal systems, can now help study the role played by cell-surface sugars and their binding proteins in plant-filamentous microbe interactions.

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Summary

Microorganisms establish symbiotic relationships with plants that range from mutualistic to pathogenic. During plant colonization, microbes secrete effector proteins that manipulate host physiology to their advantage. In turn, host plants employ receptors that recognize microbe-derived or modified-self molecules indicative of invasion. Over the past 70 years, our view on the concepts that describe the mechanisms underlying plant-microbe interactions has drastically changed (**Chapter 1**). In order to enhance our understanding on the molecular interplay between host and microbe, the work presented in this thesis was designed to further unravel components involved in the recognition of the two fungal pathogens *Verticillium dahliae* and *Cladosporium fulvum*, as well as the functions of effector proteins produced by these pathogens during tomato infection.

Chapter 2 provides an overview of the various functions displayed by effector molecules of phytopathogenic filamentous microbes, specifically their roles in the suppression of plant immune responses. However, effectors are not unique to pathogens but are employed by any microbe that encounters immune responses during plant host colonization. Moreover, plant-microbe interactions occur in environments that contain additional microbial partners, which can affect the colonizing microbe as well as the host plant. Thus, we propose that effector molecules are involved in microbial competition or cooperation in addition to their role in host manipulation, and may, therefore, shape the plant microbiome.

The carbohydrate-binding lysin motif (LysM) occurs in all living organisms, except Archaea. In fungal pathogens, effectors with varying numbers of LysMs have been implicated in the suppression of chitin-triggered immunity. The LysM effector Ecp6, which is secreted by the tomato pathogen *C. fulvum* during infection, contains three LysM domains and is able to bind chitin with high affinity. Specifically, Ecp6 disturbs chitin-induced immune responses by binding chitin with ultrahigh affinity through intramolecular LysM dimerization, resulting in its capacity to outcompete plant chitin receptors. Additionally, the singular LysM domain (LysM2), which is not involved in intramolecular chitin binding, can perturb chitin responses in tomato through a yet unknown mechanism. Due to its relatively low affinity for chitin, it has been hypothesized that LysM2 interferes with chitin receptor dimerization required for the activation of immunity. In **Chapter 3** we further investigate Ecp6 functionality in the model plant *Arabidopsis thaliana*, for which chitin perception has been intensely studied. We show that Ecp6 suppresses chitin-mediated immune responses in *A. thaliana* and affects internalization of the LysM receptor kinase AtLYK5 in a ligand-dependent manner.

All plant chitin receptors identified to date belong to the class of surface-localized LysM-containing receptor proteins. **Chapter 4** describes a chitin affinity-purification approach followed by mass spectrometry aimed at the identification of LysM receptor molecules in tomato. We identified two chitin-binding LysM receptor molecules that are closely related to *A. thaliana* LYK4 and rice *CEBiP*, respectively, both of which have been implicated in chitin

recognition in those plant species. While silencing of *SlLYK4* resulted in reduced tomato responsiveness to chitin, silencing of *SlCEBiP* had little to no effect. Since SlLYK4 carries an inactive intracellular kinase domain we hypothesize that it is involved in the formation a of heteromeric chitin receptor complex, similar to *A. thaliana* and rice. Our findings suggest that SlLYK4 is a major component of the chitin receptor complex in tomato that activates the canonical chitin signal transduction pathway.

In contrast to *C. fulvum*, *V. dahliae* infects over 200 different plant species. Like other pathogens, *V. dahliae* utilizes effector proteins to manipulate its hosts. We previously identified the effector protein Ave1, which greatly contributes to virulence during plant colonization, through comparative genomics. Ave1 homologs are ubiquitous in plants and occur in several other plant pathogens. As the distribution of the microbial homologs does not follow the phylogeny of the species they occur in, it has been hypothesized that *V. dahliae* acquired Ave1 via horizontal gene transfer. Interestingly, all homologs carry a conserved plant natriuretic peptide (PNP) sequence and *V. dahliae* Ave1 displays PNP activity (**Chapter 5**). However, complementation of a *V. dahliae Ave1* deletion mutant with microbial and plant homologs that also display PNP activity does not reinstall virulence. Thus, the contribution of *V. dahliae* Ave1 to virulence does not depend on its PNP activity. Instead, in contrast to its homologs, Ave1 additionally interacts with, and inhibits, plant endochitinases to interfere with host immunity. These findings demonstrate that Ave1 has functionally diverged from its homologs following horizontal gene transfer and evolved a novel function in plant immune suppression.

In tomato, *V. dahliae* Ave1 is recognized by the extracellular leucine-rich repeat-containing receptor protein (eLRR-RP) Ve1 resulting in resistance to *V. dahliae*. However, no function could be ascribed to the closest homolog of the Ve1 receptor, Ve2. Previous analyses of domain swap mutants between Ve1 and Ve2 have highlighted the role of the eLRR region in Ave1 ligand perception. In **Chapter 6**, we investigated the physical interaction between Ve1 and its ligand using a biochemical approach. As has been suggested previously based on the receptor mutant analyses, not only Ve1 but also Ve2 is able to bind Ave1. This binding is not solely mediated by a single region in the eLRR domain but rather requires multiple clusters of scattered eLRRs. Moreover, we show that Ave1 binding is required for the recruitment of the regulatory receptor kinase BAK1 to the Ve1 receptor complex, which represents a hallmark of eLRR-RP immune signaling.

In addition to chitin, ß-glucans are major cell wall components of filamentous microbes that elicit plant immune responses. The widespread capacity of glycan perception in plants has driven the evolution of various strategies that help filamentous microbes to evade detection. **Chapter 7** synthesizes the findings presented in this thesis and places them into the broader perspective of glycan-triggered plant immunity and the strategies that evolved in plant-associated microbes to suppress it.

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About the author

Hanna J. Rövenich was born in Berlin, Germany, on January 22nd 1988. In 2007, she began her B.Sc. studies in Biotechnology (International First Level Degree "Job Creation Oriented Biotechnology") as part of the Lifelong Learning Programme of the European Union at the University of Perugia, Italy. During the first year of her undergraduate studies, Hanna performed an internship in the Infection and Cancer Biology group of Dr. Massimo Tommasino under the daily supervision of Dr. Rosita Accardi at the International Agency for Research on Cancer in Lyon, France, where she studied the role of the transcription factor isoform ΔNp73 in Epstein-Barr virus-infected cells. In 2009, she joined the group of Dr. Sally Power for an additional internship at Imperial College London, United Kingdom, to investigate the combined impact of nitrogen and ozone on *Trifolium repens* and *Festuca ovina* as representatives of natural plant communities. To complete her B.Sc., Hanna worked on a six-months thesis project entitled "Cytokinin and dormancy in *Arabidopsis thaliana*" under the supervision of Dr. David Hanke at the University of Cambridge, United Kingdom.

In order to combine her interests in immunology and plant biology, she started her M.Sc. studies in Plant Biotechnology at Wageningen University with the specialization in Molecular Plant Breeding and Pathology. In 2011, she joined the group of Prof. Dr. Pierre de Wit for her M.Sc. thesis project. Together with Dr. Bilal Ökmen, she used a proteomic approach to identify novel effector proteins of the tomato leaf mold pathogen *Cladosporium fulvum*. Her thesis was awarded the WUF-KLV award for the best M.Sc. thesis of the academic year 2011/2012. Hanna then visited the group of Prof. Dr. Cyril Zipfel at The Sainsbury Laboratory. Under the supervision of Dr. Jacqueline Monaghan, she worked on a sensitized forward-genetic screen of *A. thaliana* mutants in the immunodeficient background *bak1-5* that led to the identification of the calcium-dependent protein kinase CPK28 and the subtilisin-like serine protease S1P as negative regulators of plant immunity upon pathogen perception mediated by surface-localized receptor kinases. In 2012, she was awarded her M.Sc. degree with distinction.

Later that year, Hanna joined the Verticillium group at the Laboratory of Phytopathology, Wageningen University, under the supervision of Prof. Dr. Bart Thomma as PhD candidate. Her research on the molecular components involved in the recognition of fungal pathogens and the strategies they evolved to evade host immunity resulted in the publication of this thesis. In 2015, she carried out a three-months stay in the group of Prof. Dr. Volker Lipka at the Georg-August-University in Göttingen, Germany, within the framework of the SUSTAIN COST Action FA1208. During her PhD, Hanna was an active member of the Wageningen PhD Council (WPC) and the PhD Council of the graduate school of Experimental Plant Sciences (EPS), which she chaired in 2014/2015. As of February 2017, Hanna is a postdoctoral researcher in the group of Prof. Dr. Alga Zuccaro at the University of Cologne, Germany, where she investigates the mechanisms underlying β-glucan recognition in plants and its importance in the establishment of mutualistic relationships between plants and fungi.

List of publications

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*equal contribution

Rovenich H., Petutschnig E.K., Valerius O., Ebert E.K., van den Berg G.C.M., Thomma B.P.H.J. (2017) A LysM receptor kinase mediates chitin-triggered defence responses in tomato. In preparation.

Rovenich H., van der Burgh A., van Damme M., Snelders N.C., Thomma B.P.H.J. (2017) Inactive tomato immune receptor homolog fails to recruit signaling components upon ligand perception. In preparation.

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†,*equal contribution

Mesarich C.H., Ökmen B., **Rovenich H.**, Griffiths S.A., Wang C., Karimi Jashni M., Mihajlovski A., Collemare J., Hunziker L., Deng C.H., van der Burgt A., Beenen H.G., Templeton M.D., Bradshaw R.E., de Wit P.J.G.M. (2017) Specific hypersensitive response-associated recognition of new apoplastic effectors from *Cladosporium fulvum* in wild tomato. Submitted.

Boshoven J.C., Ebert M.K.*, Song Y.*, **Rovenich H.**, Rojas Padilla E., Bolton M.D., Thomma B.P.H.J. (2017) Homologs of *Verticillium dahliae* effector Ave1 contribute to virulence of various fungal pathogens. Submitted.
*equal contribution

Song Y.*, Zhang Z.*, Boshoven J.C., **Rovenich H.**, Seidl M.F., Jakse J., Maruthachalam K., Liu C.-M., Subbarao K., Javornik B., Thomma B.P.H.J. (2017) Tomato immune receptor Ve1 recognizes surface-exposed co-localized N- and C-termini of *Verticillium dahliae* effector Ave1. bioRxiv 103473; DOI: doi.org/10.1101/103473.
*equal contribution

Stegmann M., Monaghan J., Smakowska E., **Rovenich H.**, Lehner A., Holton N., Belkhadir Y., Zipfel C. (2017) The receptor kinase FER is a RALF-regulated scaffold controlling plant immune signaling. *Science*, DOI: 10.1126/science.aal2541.

Kombrink A., **Rovenich H.***, Shi-Kunne X.*, Rojas Padilla E.*, van den Berg G.C.M.*, Domazakis E., de Jonge R., Valkenburg D.J., Sánchez-Vallet A., Seidl M.F., Thomma B.P.H.J. (2016) *Verticillium dahliae* LysM effectors differentially contribute to virulence on plants. *Molecular Plant Pathology*, DOI: 10.1111/mpp.12520.
*equal contribution

Rovenich H., Zuccaro A., Thomma B.P.H.J. (2016) Convergent evolution of fungal microbes towards the evasion of glycan-triggered immunity. *New Phytologist*, DOI: 10.1111/nph.14064.

Monaghan J., Matschi S., Shorinola O., **Rovenich H.**, Matei A., Segonzac C., Gro-Malinovsky F., Rathjen J.P., MacLean D., Romeis T., Zipfel C. (2014) The calcium-dependent protein kinase CPK28 buffers plant immunity and regulates BIK1 turnover. *Cell Host Microbe*, DOI: 10.1016/j.chom.2014.10.007.

Rovenich H.*, Boshoven J.C.*, Thomma B.P.H.J. (2014) Filamentous pathogen effector functions: Of pathogens, hosts and microbiomes. *Current Opinion Plant Biology*, DOI:10.1016/j.pbi.2014.05.001.
* equal contribution

Fradin E.F.*, Zhang Z.*, **Rovenich H.**[§], Song Y.[§], Liebrand T.W.H,. Masini L., van den Berg G.C.M., Joosten M.H.A.J., Thomma B.P.H.J. (2014) Functional analysis of the tomato immune receptor Ve1 through domain swaps with its non-functional homolog Ve2. *PLoS ONE*, DOI:10.1371/journal.pone.0088208.
*,[§] equal contribution

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Hanna Rövenich
Date: 29 August 2017
Group: Phytopathology
University: Wageningen University & Research

1) Start-up phase	*date*
► **First presentation of your project**	
Dissection of Ve1-mediated immunity	12 Nov 2012
► **Writing or rewriting a project proposal**	
Dissection of Ve1-mediated immunity	Oct 2012
► **Writing a review or book chapter**	
Filamentous pathogen effector functions: Of Pathogens, Hosts and Microbiomes, *Current Opinion in Plant Biology 2014, 20:96-103. doi:10.1016/j.pbi.2014.05.001*	May 2014
► **MSc courses**	
Laboratory use of isotopes	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Amsterdam, NL	30 Nov 2012
EPS PhD student day, Leiden, NL	29 Nov 2013
EPS PhD student retreat "Get2Gether", Soest, NL	29-30 Jan 2015
► **EPS theme symposia**	
EPS Theme 2: Interactions between Plants and Biotic Agents, Utrecht, NL	24 Jan 2013
EPS Theme 2: Interactions between Plants and Biotic Agents, Amsterdam, NL	25 Feb 2014
EPS Theme 2: Interactions between Plants and Biotic Agents, Utrecht, NL	20 Feb 2015
EPS Theme 2: Interactions between Plants and Biotic Agents, Leiden, NL	22 Jan 2016
► **Annual meetings (national) and other national platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	22 Apr 2013
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13-14 Apr 2015
► **Seminars (series), workshops and symposia**	
Workshops	
COST SUSTAIN workshop "Structure-guided investiagtion of effector function, action and recognition", Bucharest, Romania	10-12 Sep 2014
Workshop BU Biointeractions & Plant Health - WU Phytopathology, Wageningen, NL	10 Feb 2015
Symposia	
Plantum "Intraspecific Pathogen Variation - Implications and Opportunities", Wageningen, NL	22 Jan 2013
Mini-Symposium "How to write a world-class paper", Wageningen, NL	17 Oct 2013
Farewell symposium Pierre de Wit, Wageningen, NL	06 May 2014
Mini-Symposium Phytopathology, Wageningen, NL	24 Nov 2014
Mini-Symposium "From gene to protein and beyond", Annual Internal CLIB-GC Retreat, Bonn, Germany	22 Oct 2015
SFB1101 Symposium "Molecular encoding of plant processes", Tuebingen, Germany	04-06 Apr 2016
"Rewriting our genes" with Jennifer Doudna and Edze Westra, Wageningen, NL	30 Sep 2016

	Seminars	
	Writing for high-impact journals (Andrew Sugden, Editor of Science)	08 Feb 2013
	A. thaliana as a model system for the study of evolutionary questions (Detlef Weigel), EPS flying seminar	27 Feb 2013
	Molecular insights into spore biology and metabolism of P. infestans, the potato late blight pathogen (Howard S. Judelson), EPS flying seminar	07 May 2013
	Integrative genomics of desctructive pathogens from oomycetes to malaria parasites (Rays Jiang), EPS flying seminar	07 May 2013
	Invited seminar Brian Staskawicz	21 May 2013
	A "GoogleMAP"-type molecular view of microbes - from culture to people (Peter Dorrestein)	23 Aug 2013
	Soilborne pathogens and their natural biocontrol agents in cereal-based production systems (David Weller)	25 Sep 2013
	Endophytes in agriculture - evaluating their application via metabolomics and genomics (Ross Mann)	04 Oct 2013
	Back to the roots (Jos Raaijmakers)	07 Jan 2014
	Plant Sciences Seminar: Mutualism in Action (Toby Kiers and René Geurts)	11 Mar 2014
	From protein solution to single crystal X-ray diffraction: Chitin binding by LysM domains at atomic resolution (Jeroen Mesters)	31 Mar 2014
	Reprogramming cells for defence in plant innate immunity (Jane Parker)	09 Apr 2014
	Plant Metacaspases (Frank van Breusegem)	09 Apr 2014
	Dissecting the interactions between Phytophthora sojae and soy bean: making sense of signaling and effectors (Yuanchao Wang)	16 Jul 2014
	Evolution of plant-herbivore interactions: insights from genomics (Noah Whiteman)	17 Jul 2014
	Chromatin structure controls centromeres and secondary metabolism in filamentous fungi (Michael Freitag)	21 Oct 2014
	Genetics and epigenetics: a complex relationship (Ortrun Mittelstein-Scheid)	19 Nov 2014
	The evolutionary significance of gene and genome duplications (Yves van den Peer)	03 Feb 2015
	How Ralstonia solanacearum succeeds in plant xylem vessels (Caitilyn Allen)	29 Apr 2016
	Effectors as molecular probes to understand pathogenesis (Wenbo Ma)	20 Jun 2016
►	**Seminar plus**	
	Michael Freitag, Oregon State University, USA	21 Oct 2014
	Caitilyn Allen, University of Wisconsin-Madison, USA	29 Apr 2016
►	**International symposia and congresses**	
	Keystone Symposium: Plant Immunity - Pathways and Translation, Big Sky, MT, USA	07-12 Apr 2013
	5th PhD retreat (EPSR), Ghent, Belgium	23-26 Jul 2013
	28th Fungal Genetics Conference, Pacific Grove, CA, USA	17-22 Mar 2015
	12th New Phytologist workshop "The apoplast as battleground for plant-microbe interactions", Rauischholzhausen, Germany	09-10 Jul 2015
►	**Presentations**	
	Talks	
	Functional analysis of the fungal effector Ave1, Spring school 'Host-microbe interactomics', Wageningen, NL	03 Jun 2014
	Molecular mechanisms underlying the Verticillium-tomato interaction, COST SUSTAIN workshop, Bucharest, Romania	10 Sep 2014
	Work on effectors and receptors in Phytopathology, Workshop BU Biointeractions & Plant Health - WU Phytopathology, Wageningen, NL	10 Feb 2015
	The Verticillium effector Ave1 contributes to fungal virulence through inhibition of plant chitinases, ALW meeting 'Experimental Plant Sciences', Lunteren, NL	14 Apr 2015

	Fungal strategies to subvert host chitin-triggered immunity, Seminar CEPLAS (Groups Doehlemann and Zuccaro), Institute for Genetics, University of Cologne, Cologne, Germany	18 Sep 2015
	Fungal strategies to subvert host chitin-triggered immunity, Mini-Symposium, Annual Internal CLIB-GC Retreat, Bonn, Germany	22 Oct 2015
	Posters	
	The tomato receptor-like protein Ve1 interacts with the fungal effector Ave1 to initiate immune signaling, Keystone Symposium, MT, USA	10 Apr 2013
	The fungal effector Ave1 is a putative inhibitor of plant chitinases, COST SUSTAIN workshop, Bucharest	10 Sep 2014
	The Verticillium effector Ave1 contributes to fungal virulence through inhibition of plant chitinases, 28th Fungal Genetics Conference, Pacific Grove, CA, USA	20 Mar 2015
	The Verticillium effector VdAve1 is a dual function protein, SFB1101 symposium, Tuebingen, Germany	04 Apr 2016
►	**IAB interview**	
►	**Excursions**	
	Subtotal Scientific Exposure	*24.9 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	PhD course: Introduction Bioinformatics 'A User's Approach', Wageningen, NL	27-31 Aug 2012
	PhD course: Advanced proteomics, Wageningen, NL	23-26 Apr 2013
	PhD spring school 'Host-microbe interactomics', Wageningen, NL	02-04 Jun 2014
►	**Journal club**	
	Member of the Verticillium literature discussion group of Phytopathology	2012-2016
►	**Individual research training**	
	Short term scientific mission (COST FA1208 SUSTAIN Action) at the Schwann-Schleiden Research Centre, Department of Plant Cell Biology, Laboratory of Prof. Volker Lipka, Göttingen, Germany	07 Sep-30 Nov 2015
	Subtotal In-Depth Studies	*9.6 credits**

4) Personal development		*date*
►	**Skill training courses**	
	PhD Competence Assessment	May-Jun 2013
►	**Organisation of PhD students day, course or conference**	
	ExPectations Day "Communication and ethics in science"	28 Mar 2014
	Get2Gether PhD Student Retreat, Soest, NL	29-30 Jan 2015
►	**Membership of Board, Committee or PhD council**	
	Member EPS PhD council	Mar 2013- Apr 2014
	Chair of the EPS PhD council	Apr 2014-Apr 2015
	Subtotal Personal Development	*5.4 credits**

TOTAL NUMBER OF CREDIT POINTS*	**53.4**

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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