Functional analysis of LysM effectors secreted by fungal plant pathogens

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Chapter 1

General introduction and outline of the thesis

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Abstract

Despite the deployment of antifungal defence strategies, fungal diseases occur in all types of multicellular organisms. In plants, the role of fungal chitin as pathogen-associated molecular pattern that activates host defence is well established. Interestingly, plants employ homologs of the chitin immune receptors to initiate microbial symbiosis. Accumulating evidence shows that fungal pathogens developed secreted effectors to disarm chitin-triggered host immunity.

Introduction

Together with cellulose, a D-glucose homopolymer that constitutes the primary structural component of plant cell walls, chitin is the most abundant carbohydrate found in nature. Chitin is an *N*-acetyl-D-glucosamine (GlcNAc) homopolymer that is found as primary structural component in the cell walls of fungi, the exoskeleton of arthropods and egg-shells of nematodes.

Fungal cell walls are largely composed of carbohydrate polymers that include β-glucans, chitin and mannans in addition to glycoproteins. While the three carbohydrate components are interspersed throughout the cell wall, chitin presumably localizes near the plasma membrane, whereas the mannans are thought to line the outer cell wall (Bowman, 2006; Lenardon et al., 2010). Importantly, extensive differences in cell wall composition occur not only between fungal species, but even between strains of the same species and between morphological structures of the same strain (Latgé et al., 2007; 2010).

Fungi are the most important plant pathogens that cause significant yield losses worldwide (Agrios, 2005). Similar to animals, also plants possess an innate immune system to sense the presence of microbial invaders by means of receptors that detect microbial-derived molecules, or by receptors that detect plant-manipulating activities of pathogens (Boller and Felix, 2009). As plants do not contain chitin, this molecule is recognized as a non-self component and activates host immune responses (Felix et al., 1993; Shibuya et al., 1993).

Plant defence against microbial infections

The first obstacles in plants against pathogen attack are formed by physical, enzymatic and chemical barriers that are constitutively present and include the cuticle, a waxy layer that is deposited on the plant surface, the plant cell wall and anti-microbial compounds that include enzymes, peptides and secondary metabolites (Osbourn et al., 1996; Broekaert et al., 1997). In addition to these preformed defences, inducible defence mechanisms can be activated upon recognition of pathogen attack (Dixon et al., 1994; Thomma et al., 2001). These include structural fortifications at the site of attempted pathogen ingress and the

production and release of antimicrobial molecules at the site of infection as well as in tissues away from the initial infection site. Furthermore, a localized apoptosis-like hypersensitive cell death response may occur at the infection site. Eventually this all can result in systemic acquired resistance (SAR), a long-lasting (weeks to months) state of induced immunity against a broad range of pathogens (Ryals et al., 1996).

Although over-simplified (Thomma et al., 2011), the current view of the evolution of inducible defence responses in plant pathogen interactions is nicely captured in the so-called zig-zag model (Jones and Dangl, 2006). In this model, the first inducible defences are activated by pattern recognition receptors (PRRs), which are cell surface receptors that recognize microbial-associated molecular patterns (MAMPs; also referred to as pathogen-associated molecular patterns or PAMPs) as non-self components; so-called MAMP-triggered immunity (MTI; also referred to as PAMP-triggered immunity or PTI; Fig. 1). This defence response includes local cell wall fortifications, production of reactive oxygen species (ROS), and the production and release of antimicrobial compounds, which collectively will stop most microbial invaders. The key element of the zig-zag model is the notification that successful pathogens are able to overcome MTI by the use of secreted effectors that

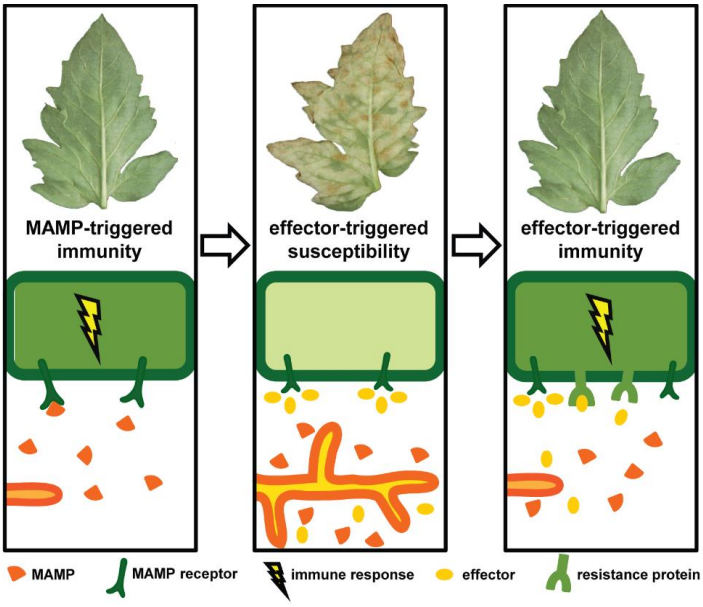

Figure 1. Evolutionary development of the interaction between fungal pathogens (in yellow) and their host plants (in green). Perception of pathogen derived MAMPs by plant MAMP receptors on the plant cell membrane triggers defence responses leading to MTI (1st panel). Successful pathogens evolved to produce effector proteins that perturb MTI and the host plant will get diseased, this is known as ETS (middle panel). In turn, the evolution of plants to produce resistance proteins that recognize these effectors enables activation of defence responses and results again in resistant plants or ETI (right panel).

perturb host defences in a pro-active manner, thus establishing effector-triggered susceptibility (ETS; Fig. 1) (Jones and Dangl, 2006; Panstruga and Dodds, 2009; de Jonge et al., 2011). Many of these effectors appear to have molecular targets inside host cells. While pathogenic Gram negative bacteria typically inject such effectors directly into the host cytoplasm by means of their type-III secretion machinery, fungal effectors carry host cell uptake motifs that mediate autonomous translocation into the host cytoplasm (Panstruga and Dodds, 2009; de Jonge et al., 2011). It has been shown that some effectors directly target and destabilize MAMP receptor complexes (Shan et al., 2008; Gimenez-Ibanez et al., 2009). So far, however, the molecular targets of most fungal effectors remain enigmatic (de Jonge et al., 2011).

During evolution, plants have evolved to intercept the activity of particular pathogen effectors through novel receptors that are typically called resistance proteins. Although several resistance proteins have been characterized as cell surface receptors, the majority of these receptors are cytoplasmic proteins of the nucleotide binding leucine-rich repeat (NB-LRR) type that again activate inducible host defences, generally referred to as effector-triggered immunity (ETI; Fig. 1). It was initially proposed that defence responses associated with ETI occur more quickly, are more prolonged and stronger than MTI responses, and generally include the hypersensitive cell death, an apoptosis-like programmed cell death response at the site of attempted penetration (Jones and Dangl, 2006). However, it rather appears that ETI and MTI both can be robust or weak, depending on the specific interaction between effector and receptor, and possibly also on environmental conditions (Thomma et al., 2011). In any case, ETI halts pathogen ingress and poses a selection pressure on the pathogen to either lose or mutate the effector that is recognized to avoid the activation of host immunity (Joosten et al., 1994), or to acquire novel effectors to suppress the ETI response (Houterman et al., 2008). This arms race will continue because new plant receptors will evolve that recognize either the mutated or the newly acquired effectors to again activate ETI (Jones and Dangl, 2006).

According to the zig-zag model, MTI is generally triggered by a wide range of microbes because MAMPs are conserved throughout classes of microbes, whereas the propensity to trigger ETI is typically pathogen strain-specific (Jones and Dangl, 2006). However, it is increasingly being appreciated that the distinction between MAMPs and effectors, and therefore between MTI and ETI, cannot strictly be maintained, and that there is a continuum between MTI and ETI (Thomma et al., 2011).

Chitin as target and trigger of immune responses

Plants produce a wide array of pathogenesis-related (PR) proteins with antimicrobial activity to defend themselves against microbial invaders. Some of these PR proteins are hydrolytic enzymes that target fungal cell walls, such as chitinases and glucanases. Chitinases have been shown to contribute to defence against fungi in two distinct manners. Exochi-

tinases reside in the apoplast and are not considered to be detrimental to fungal growth, but are presumed to be able to release short-chain chitin oligosaccharides from fungal cell walls. These chitin oligosaccharides act as MAMPs that are detected by host receptors and activate MTI (Felix et al., 1993; Shibuya et al., 1993). These responses include the production of endochitinases that accumulate in the vacuole and are released upon cellular collapse to act as powerful antifungals due to their fungal cell wall hydrolytic activity (Schlumbaum et al., 1986; Grison et al., 1996; Nishizawa et al., 1999).

Chitin receptors of the immune system

By now, several MAMP receptors have been cloned from plants, including receptors that are critical for chitin-triggered immune responses. Through cross-linking of plasma membranes with radio-labeled chitin, the presence of a chitin receptor was revealed in rice (*Oryzae sativa*) (Ito et al., 1997). Subsequently, a 75 kDa protein was isolated, cloned and characterized as Chitin Oligosaccharide Elicitor-Binding Protein (CEBiP) (Kaku et al., 2006). Knockdown of *CEBiP* expression resulted in a significant reduction of chitin-induced defence responses, such as the production of reactive oxygen species and the expression of chitin-regulated genes, and allowed increased spread of the fungal pathogen *Magnaporthe oryzae* that causes the devastating rice blast disease (Kaku et al., 2006; Kishimoto et al., 2010). These results demonstrate that chitin perception by the CEBiP receptor is critical for defence against fungal attack in rice plants. The CEBiP protein was found to contain lysin motifs (LysMs) in the extracellular domain that are assumed to be responsible for chitin binding. Since CEBiP lacks intracellular signalling domains, additional molecular components are likely required to activate cellular signalling leading to chitin-triggered defence responses (Kaku et al., 2006). Recently a LysM-containing cell surface receptor with a cytoplasmic kinase domain, the Chitin Elicitor Receptor Kinase-1 (OsCERK1) was identified. Together with CEBiP, OsCERK1 was shown to be required for chitin signalling in rice cells, suggesting that CEBiP and OsCERK1 participate in a chitin-induced receptor complex on the rice plasma membrane (Shimizu et al., 2010).

LysM containing cell surface receptors not only play a critical role in chitin-triggered defence against fungi in rice. Prior to the identification of OsCERK1, a homologous receptor was identified in the genetic model weed Arabidopsis (*Arabidopsis thaliana*), designated AtCERK1 (Miya et al., 2007; Wan et al., 2008). AtCERK1 was shown to bind chitin (Petutschnig et al., 2010; Iizasa et al., 2010), and is required for chitin-triggered immunity against fungal attack (Miya et al., 2007; Wan et al., 2008). Since ectodomain truncations in which one or two LysM domains were deleted no longer bind chitin, it has been suggested that all three LysM domains are required for chitin binding. Moreover, it was shown that chitin binding rapidly induces phosphorylation of the CERK1 juxtamembrane and kinase domain *in vivo* and it was shown that the kinase activity of CERK1 is required for its chitin-triggered immunity (Petutschnig et al., 2010).

Recently, a receptor homologous to rice CEBiP was shown to contribute to defence against *Magnaporthe oryzae* in the monocotyledonous crop barley (Tanaka et al., 2010) and in *Medicago trunculata* one of the two LysM proteins identified was demonstrated to be membrane-bound and to have chitin-binding activity, suggesting a similar role in MAMP perception and defence response (Fliegmann et al., 2011). Thus, LysM containing cell surface receptors that play a role in chitin-triggered immunity in plants are conserved and ancient, as they are broadly distributed in vascular plants, including monocotyledenous (such as rice) and dicotyledonous (such as Arabidopsis) plants that diverged at least 140 million years ago, but do not occur in algae or mosses (Fliegmann et al., 2011).

The role of LysMs in chitin binding

Despite the fact that several receptors that are critical for chitin signalling were shown to carry LysM domains and bind chitin, no evidence is presented yet that these receptors bind chitin actually through their LysM domains. LysMs were originally identified in peptidoglycan-hydrolyzing phage lysozymes (Garvey et al., 1986) and similarly occur in various bacterial hydrolases that were shown to bind to peptidoglycan (Eckert et al., 2006; Buist et al., 2008). Peptidoglycan is a bacterial cell wall polymer that is composed of alternating *N*-acetylmuramic acid and GlcNAc residues, the latter of which is also the chitin monomer. In addition to chitin receptors, LysMs also occur in various other types of chitin-binding proteins, such as chitinases (Buist et al., 2008). The canonical three-dimensional LysM domain structure is an αββα fold in which the two α-helices are packed against one side of the double-stranded antiparallel β-sheet (Bateman and Bycroft, 2000). First evidence for the role of LysM domains in direct binding of chitin was reported by Ohnuma and collaborators (2008) based on NMR spectroscopy experiments. The two LysM domains of the fern *Pteris ryukyuensis* chitinase A were shown to physically interact with (GlcNAc)5 in a 1:1 stoichiometry and the recognition site was identified as a shallow groove on the surface of the LysM domain. Recently, the LysM domain of a type III CVNH lectin from the rice blast fungus *M. oryzae* was demonstrated to tightly bind to oligosaccharides of 5 or 6 GlcNAc residues using NMR (Koharudin et al., 2011). In both studies it was determined that the highly conserved region between α-helix 1 and β-strand 1 and the loop between α-helix 2 and β-strand 2 critically contribute to the specificity and strength of carbohydrate binding (Ohnuma et al., 2008; Koharudin et al., 2011). However, additional experimental evidence is still needed in order to elucidate the specificity, the affinity and the biological function of this recognition.

Fungal effectors interfere with chitin-related immune responses

Since the zigzag model proposes that the intrinsic function of effectors is to interfere with MTI responses in order to establish host susceptibility, the existence of fungal effectors

that interfere with chitin-triggered immunity can be anticipated. The fungal pathogen *Cladosporium fulvum* is the causal agent of leaf mould of tomato, and due to its particular colonization style, *C. fulvum* has been successfully used as a model pathogen for decades (Thomma et al., 2005; Joosten and de Wit, 1999). The fungus penetrates tomato leafs through stomates, natural openings on the surface for gas exchange, and colonizes the so-called apoplastic space in between the mesophyll cells that constitute the interior of a leaf without penetrating plant cells (Fig. 2). Therefore, molecular components of the pathogen and of the host that are involved in the establishment of the interaction have to pass the apoplastic space and can be isolated relatively easy by washing out this space.

The *Cladosporium fulvum* Avr4 effector

Through the characterization of intercellular washing fluids, several *C. fulvum* effector proteins have been identified, one of which is designated Avr4 (Joosten et al., 1994). Avr4 is encoded as a pre-pro-protein of 135 amino acids that includes a signal peptide and eight cysteines. Interestingly, binding studies showed that this effector specifically binds to fungal cell walls, and not to those of tomato (Westerink et al., 2002). Based on structural analysis, it was subsequently found that Avr4 harbors an invertebrate chitin binding domain (van den Burg et al., 2003, 2004). Through its binding to chitin, Avr4 can protect fungal hyphae against hydrolysis by chitinases during plant colonization because the fungal cell wall is shielded and no longer accessible to these lytic enzymes (Fig. 3) (van den Burg et al., 2006). In addition, Avr4 binding to *C. fulvum* hyphae may also reduce the release of

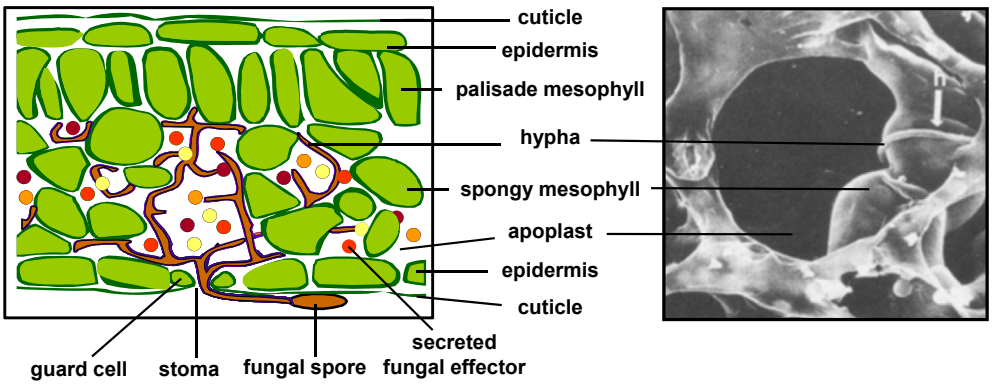

Figure 2. Schematic vertical dissection of a plant leaf and a close-up scanning-electron microscope picture both showing the tomato apoplast during colonization of *C. fulvum*. Interaction between *C. fulvum* and its host takes place in the apoplast were effector proteins are secreted to establish infection (indicated as coloured spheres). (The SEM picture is taken from: de Wit, P.J.G.M. Light and scanning-electron microscopic study of infection of tomato plants by virulent and avirulent races of *Cladosporium fulvum*. Neth. J. Plant Pathol. 83 (1977) 109–122, with permission).

chitin MAMPs fragments that trigger host immune responses. In accordance with these functions in fungal virulence, it was demonstrated that Avr4 is a genuine virulence factor, as compromised *Avr4* expression significantly affected *C. fulvum* virulence (van Esse et al., 2007).

The *Cladosporium fulvum* LysM effector Ecp6

The most recently identified *C. fulvum* effector is one of the most abundant proteins found in the apoplast of infected tomato plants and was designated extracellular protein 6 (Ecp6) (Bolton et al., 2008). Whereas other *C. fulvum* effectors are characterized by absence of sequence homology and absence of recognizable protein domains, Ecp6 was found to harbor three LysM domains. Ecp6 was shown to contribute to virulence as silencing of Ecp6 in *C. fulvum* compromised aggressiveness on tomato (Bolton et al., 2008). Based on the presence of LysM domains in the protein sequence, the ability of Ecp6 to bind chitin was tested. Indeed, Ecp6 was found to bind chitin in affinity precipitations with insoluble chitin *in vitro* (de Jonge et al., 2010). Moreover, with glycan array analysis to test the affinity to more than 400 different glycan substrates it was demonstrated that Ecp6 only bound to chitin oligosaccharides. Initially, Ecp6 was tested for the ability to protect fungal hyphae against chitinases as described for Avr4, but Ecp6 failed to protect hyphae against hydrolysis by chitinases (de Jonge et al., 2010). Subsequently, the hypothesis was tested whether Ecp6 interferes with the perception of chitin oligosaccharide MAMPs. It has previously been demonstrated that chitin induces a pH-shift in tomato cell suspensions, indicative of an immune response (Felix et al., 1993). Pre-incubation of chitin with Ecp6, however, largely abolished this pH-shift, as well as other chitin-induced responses in cell suspensions and in plant tissues, suggesting that Ecp6 is able to suppress the activation of host immune responses by chitin (de Jonge et al., 2010). Consistent with a role as suppressor of immune activation by scavenging chitin MAMPs, direct competition for binding of chitin with plant chitin receptors was anticipated. Because the tomato chitin receptor is not identified thus far, competition of Ecp6 with the rice receptor CEBiP was evaluated. Competition assays in which chitin oligosaccharides and Ecp6 were incubated with rice membranes that contain the CEBiP receptor demonstrated that Ecp6 was able to sequester the chitin oligosaccharides and prevented chitin binding to the receptor (de Jonge et al., 2010). These experiments show that chitin triggered immunity can be prevented by the fungal effector Ecp6 because it sequesters chitin MAMPs (Fig. 3).

Chitin binding effectors of other fungal pathogens

Pathogen effector proteins are typically lineage-specific, implying that they are typically not conserved and that pathogen species, and sometimes even strains, have highly divergent effector catalogs (de Jonge et al., 2011). Intriguingly, homologs of the *C. fulvum*

effector Avr4 have recently been identified in other fungi, such as *Mycosphaerella fijiensis*, the causal agent of the devastating black Sigatoka disease of banana (Stergiopoulos et al., 2010). The *M. fijiensis* Avr4 homolog was found to protect fungal hyphae against chitinase activity in a similar fashion as *C. fulvum* Avr4. Like *C. fulvum*, all other fungi that harbor Avr4 homologs belong to the class of Dothideomycetes. However, not all Dothideomycete fungi contain Avr4 homologs, as they were not found in the genome sequence of the wheat pathogen *Mycosphaerella graminicola*, causal agent of leaf blotch of wheat, for instance.

Whereas Avr4 homologs are not found outside the class of Dothideomycetes (Stergiopoulos et al., 2010), Ecp6 homologs widely occur in the fungal kingdom (Bolton et al., 2008; de Jonge and Thomma, 2009). These homologs have been termed LysM effectors (de Jonge and Thomma, 2009). Although the sequence homology within the LysM effector family is low, all these LysM effectors have in common that they are predicted to be secreted, contain a varying number of LysM domains and lack other recognizable protein domains (de Jonge and Thomma, 2009). Many fungal genomes contain multiple genes that encode LysM effectors with varying numbers of LysM domains. The genome of *M. graminicola* contains three putative LysM effector genes, two of which are expressed during infection (Marshall et al., 2011). Of these two, the LysM effector Mg3LysM contains three LysM domains, similar to *C. fulvum* Ecp6, while the Mg1LysM effector contains only one LysM domain. Interestingly, both LysM effectors were found to bind chitin *in vitro*. However, whereas Mg3LysM was able to suppress chitin-triggered host defences in a similar fashion

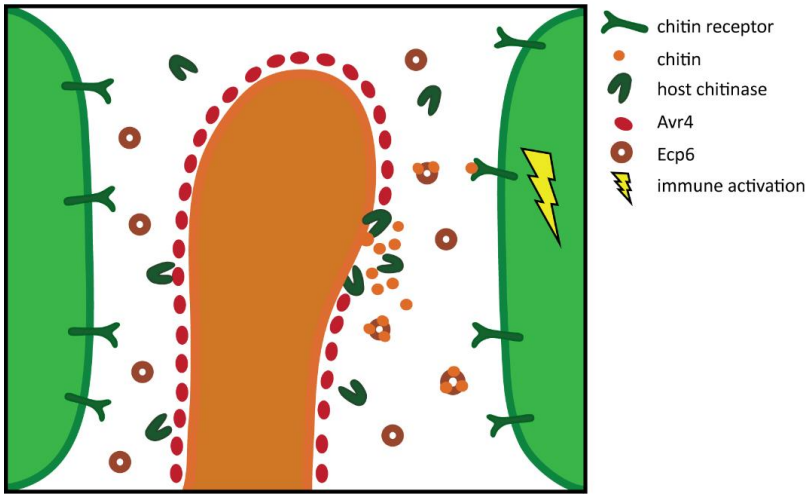

Figure 3. The role of chitin-binding *C. fulvum* effectors Avr4 and Ecp6 in fungal virulence. Avr4 protects the fungal cell wall chitin from hydrolysis by plant chitinases. Chitin oligosaccharides are released into the apoplast, when Avr4 protection is not sufficient but also at hyphal growing sites, and upon their perception by the plant MTI would be triggered. However, to prevent activation of host chitin receptors, free chitin oligosaccharides are sequestered by Ecp6.

as *C. fulvum* Ecp6, Mg1LysM failed to provide such protection. In agreement with this observation, infection studies with knock-out mutants showed that Mg3LysM contributes to virulence of the fungus, as knock-out mutants for this gene displayed reduced virulence on wheat and triggered stronger host defence responses, whereas a similar contribution to virulence could not be demonstrated for Mg1LysM (Marshall et al., 2011). Interestingly, however, Mg3LysM displayed a dual function in virulence as, in addition to its ability to suppress chitin-triggered host immunity in a similar fashion as *C. fulvum* Ecp6 (Fig. 3), Mg3LysM also protected fungal hyphae against hydrolysis by plant chitinases in a similar fashion as *C. fulvum* Avr4 (Fig. 3) (Marshall et al., 2011). Furthermore, also Mg1LysM displayed the ability to protect fungal hyphae against chitinase hydrolysis. The finding that this particular activity is displayed by both LysM effectors may explain why *Mg1LysM* knockout transformants did not display reduced virulence during infection of wheat plants. Intriguingly, no *C. fulvum* Avr4 homologs were identified in the *M. graminicola* genome, which may explain why, in contrast to *C. fulvum* Ecp6, LysM effectors of *M. graminicola* may have evolved to protect fungal hyphae against plant hydrolytic enzymes (Marshall et al., 2011). These findings demonstrate that LysM effectors can contribute to fungal virulence through various mechanisms. Further research involving more family members will provide more insight in the variable roles that LysM effectors play in establishing fungal infections.

LysM receptors in symbiosis

Interestingly, plant LysM receptors are not exclusively involved in the detection of pathogenic micro-organisms to mount an immune response, but also in detection of beneficial micro-organisms such as mycorrhizal fungi and rhizobia bacteria to establish a symbiosis (Knogge and Scheel, 2006; Gough and Cullimore, 2011). When plants started to colonize land, some 400 million years ago, mycorrhizal symbiosis evolved and presently mycorrhizae occur in a broad range of habitats and in the majority of plant species. Most widespread are arbuscular mycorrhiza (AM), covering ~150 species of the Glomeromycota phylum, that colonize 70-90% of the plant species (Smith and Read, 2008). During colonization, AM hyphae form tree-like structures (arbuscules) that invaginate the plasma membrane of plant cells to connect the plant with an extensive hyphal network that provides the plant with water and nutrients while the fungus obtains carbohydrates in return. In contrast to the wide range of plant species that are hosts for AM fungi, bacterial endosymbiosis is essentially restricted to plant species of the legume family, and concerns a variety of phylogenetically diverse Gram negative bacteria termed rhizobia (Masson-Boivin et al., 2009). The symbiosis with rhizobia results in the formation of root nodules containing bacteria that are able to fix atmospheric nitrogen. Rhizobial symbiosis evolved more recently than AM symbiosis and is thought to have emerged some 60 million years ago (Sprent and James, 2007).

Regulation of symbiosis through chitin-related molecules and LysM receptors

To establish symbiosis the host plant needs to undergo physiological changes. A number of the signalling genes that control rhizobial symbiosis are also involved in the development of AM symbiosis, and define the so-called common symbiotic pathway (CSP) (Oláh et al., 2005; Parniske, 2008). The key symbiotic signals produced by rhizobial bacteria to initiate symbiosis are lipo-chitooligosaccharides (LCOs), called nodulation (Nod) factors. These molecules contain a GlcNAc backbone of four to five residues that are decorated with side groups to allow specificity of the interaction between different rhizobial species and receptors of their specific legume hosts (Hamel and Beaudoin, 2010). Because rhizobial symbiosis appeared much later in evolution and part of the signalling pathway is shared with the AM symbiosis pathway, it has been suggested that rhizobial symbiosis has evolved from AM symbiosis, and that Nod-factors are derived from the AM secreted signalling molecule, the putative Myc factor, possibly through horizontal gene transfer from myccorhiza to rhizobia (Moulin et al., 2001). Indeed, the AM species *Glomus intraradices* was recently found to secrete a mixture of lipo-chitooligosaccharide Myc factors that stimulate AM formation in plant species of diverse families (Maillet et al., 2011).

The related structure of Nod and Myc factors suggest that perception by plant hosts occurs via similar receptor proteins. Extensive research led to the identification of various legume Nod factor receptors (NFRs) in the legume model species *Medicago truncatula* and *Lotus japonicus* (Limpens et al., 2003, Madsen et al., 2003, Radutoiu et al., 2003). All these receptors were found to contain LysMs in their ectodomains, although presently binding of Nod factors by these LysM receptors has not been demonstrated. Using the LysM domain structure of the *Escherichia coli* membrane-bound lytic murein transglycosidase D (MltD), homology modeling of the three LysM domains of *M. truncatula* NFP was performed and an αββα structure was similarly proposed for each LysM domain of MtNFP (Mulder et al., 2006). One single Nod factor binding site was predicted for each LysM domain at the loops between α-helixes and β-strands, which are less conserved regions in the LysM domains (Mulder et al., 2006). Genetic experiments strongly suggest that the divergent LysM domains of the receptors play a critical role in the discrimination of different Nod factors that are characteristic of their corresponding symbionts (Radutoiu et al., 2007). Intriguingly, some trees that belong to the non-legume genus *Parasponia* also form rhizobial symbiosis. A LysM receptor that was recently identified in *Parasponia andersonii*, designated PaNFP, was shown to have a role in AM as well as rhizobial symbiosis, which confirms that the perception of lipo-chitooligosaccharides plays an essential role also in AM symbiosis and that the same receptor is involved in establishment of AM and rhizobial symbioses (Op den Camp et al., 2010).

Defence or symbiosis?

The structural similarity of signalling molecules and plant receptors in chitin signalling and in symbiosis suggests that symbiotic responses and chitin induced defence responses are evolutionary linked. Moreover, the close phylogenetic relationship between Nod factor LysM receptors and CERK1 suggests that Nod factor perception in legumes evolved from the more widely conserved chitin perception (Zhang et al., 2007; Lohmann et al., 2010; Gough and Cullimore, 2011). As a consequence, legume plants are predicted to have chitin and Nod factor receptors within the same family, and it was recently demonstrated that the LysM receptor-like kinase family in the legume Lotus japonicus encompasses 17 related members, including three chitin-inducible members that were proposed as candidate chitin receptors (Lohmann et al., 2010). Several examples demonstrate the close relationship, and even partial overlap, between symbiotic and defence responses. For example, chitin was shown to induce symbiotic genes independent of *NRF1* (Nakagawa et al., 2011). Conversely, Nod factors can activate *NFR1*-dependent expression of host defence signalling (Nakagawa et al., 2011), and host defence is induced in *Nicotiana benthamiana* leaves upon over-expression of Nod factor receptors (Madsen et al., 2011). Finally, micro-array analysis of pathogen and AM infected rice plants has shown transcriptional overlap between the two responses (Güimil et al., 2005).

A recent study highlights a surprisingly large overlap in the phosphoproteome of the legume *Lotus japonicus* within minutes after perception of nodulation factor or flagellin peptide, a bacterial derived MAMP that triggers host defence (Serna et al., 2011). However, in addition to the overlap, signal-specific responses were observed as NFR1 and NFR5 were only required for nodulation factor-induced phosphoproteome changes. Moreover, only flagellin peptide induced an oxidative burst and MAP kinases that are considered as early defence response markers (Serna et al., 2011). Also, signalling induced through chitin receptors and Nod factor receptors is distinct, as mutation of legume Nod factor receptors does not prevent chitin-dependent induction of host defence (Wan et al., 2008). Indeed, the intracellular kinase domains of the chitin LysM receptor CERK1 and the Nod factor receptor NRF1 from *Lotus japonicus* show high homology but induce either defence or symbiotic responses, respectively (Nakagawa et al., 2011). Functional analysis of a chimeric receptor with the extracellular NRF1 domain and the CERK1 kinase domain revealed that substitution of a few amino acids of the CERK1 kinase domain for NRF1 amino acids can lead to the activation of symbiotic responses. This shows that, despite the high overall homology, discrimination between defence and symbiotic signalling occurs through the kinase domain of the respective LysM receptors.

The size of the Nod factor and the chitin oligosaccharide might also be involved in specificity of LysM receptors for ligand perception, as Nod factors are generally based on 4 or 5 GlcNAc residues while the most active chitin elicitors have larger degrees of polymerisation (Shibuya and Minami, 2001; Yamada et al., 1993, Hamel and Beaudoin, 2010). Further-

more, Nod factors typically require several modifications, including sulfation, acetylation, and acylation (D'Heaze and Holsters, 2002; Hamel and Beaudoin, 2010) and are active at concentrations down to 10-11M, whereas chitin elicitors are typically used at concentrations down to 10-9M (Gough and Cullimore, 2011). This suggests that the affinity of Nod factor receptors for their ligands is higher than the affinity of chitin receptors for chitin.

Concluding remarks

LysM receptors are involved in the perception of fungal cell wall derived chito-oligosaccharides and elicit defence responses and fungal resistance in plants. This perception system has high homology to the perception by plant LysM receptors of lipo-chitooligosaccharide Nod factors that are secreted by symbiotic rhizobia bacteria. The discovery of fungal lipo-chitooligosaccharide signals and a LysM receptor required for AM symbiosis leads to questions on how plants discriminate pathogens from symbionts, how plants recognize different types of symbionts, and how these different perception mechanisms evolved. This is particularly relevant for AM fungi that, despite being symbiotic microorganisms, also contain chitin in their cell walls. It can be speculated that they avoid host defence induction through poor accessibility of their chitin, possibly mediated by LysM effectors, or through the production of chitin deacetylases that convert chitin into chitosan which is a poor inducer of host defence responses (Gough and Cullimore, 2011). Alternatively, they have highly specialized effectors to intercept host defence signalling.

Also mammals are frequently exposed to chitin; during invasion of chitin-containing pathogens such as fungi and nematodes, or through inhalation or digestion of chitin molecules derived from, for example, dust mites and arthropods. Various studies show that a range of mammalian cell types are able to recognize chitin and react with an immune response (Shibata et al., 1997a; 1997b; Reese et al., 2007; Lee et al., 2008; Da Silva et al., 2009). These immune responses seem to depend on the size of the chitin fragments used, as large chitin fragments (>100 μm) are immunologically inactive, while intermediate (40-70 μm) and small (<40 μm) fragments have been reported to activate cytokine production via a range of pattern recognition receptors in macrophages as part of an inflammatory response (Da Silva et al., 2008; 2009; Reese et al., 2007; Lee et al., 2008; Lenardon et al., 2010). However, other studies contradict these findings by showing that chitin does not directly stimulate production of cytokines from peripheral blood mononuclear cells and macrophages (Mora-Montes et al., 2011). Moreover, it was found that chitin blocked the recognition of *C. albicans* by these cells (Mora-Montes et al., 2011). Presently, several chitin binding proteins, including Ym1, the C-type lectin RegIIIg, the fibrinogen C domain-containing protein1 FIBCD1, chitinases and the mannose receptor, have been identified, although their role in fungal recognition still remains unclear (Da Silva et al., 2009; Lee et al., 2008; Schlosser et al., 2009; Latgé 2010; Mora-Montes et al., 2011). Interestingly, also the genomes of mammalian fungal pathogens contain LysM effector genes, suggesting

that also these pathogens might sequester chitin to prevent recognition via putative LysM effectors in their hosts (de Jonge and Thomma, 2009). Thus, the role of chitin detection may be similar in the interactions of plant and of mammalian fungal pathogens with their respective hosts.

Thesis outline

After the identification and characterization of *C. fulvum* Ecp6, LysM effector genes were found to occur in many fungal species. This thesis describes a functional analysis of LysM effectors that are secreted by various plant pathogenic fungi.

In **Chapter 2** the characterization LysM effectors of three fungal plant pathogens is described: Slp1 from *Magnaporthe oryzae*, Mg1LysM and Mg3LysM from *Mycosphaerella graminicola* and ChELP1 and ChELP2 from *Colletotrichum higginsianum*. The LysM effectors were heterologously produced in *Pichia pastoris* and tested for chitin-binding and their ability to suppress chitin-triggered immunity. In addition, it was tested whether the LysM effectors have the ability to protect fungal hyphae against plant-derived cell wall hydrolytic enzymes, which are secreted by plant hosts to stop fungal growth. *M. graminicola* Mg3LysM was demonstrated to protect fungal hyphae against such hydrolytic enzymes, whereas *C. fulvum* Ecp6 does not have this capacity. Mg3LysM and *C. fulvum* Ecp6 both contain three LysM domains and show a high overall similarity. In **Chapter 3** it was further investigated how the ability to protect against hydrolytic enzymes is established by Mg3LysM. To this end, a series of chimeric proteins, in which LysM domains of Mg3LysM were swapped with the corresponding LysM domains of Ecp6, were functionally analysed.

In **Chapter 4** LysM effectors of soil-borne vascular pathogen *V. dahliae* were investigated. Four LysM effector genes were characterized in strain JR2 that are also found in the core genome of eleven other sequenced *V. dahliae* strains. Interestingly, an additional VdLysM effector gene (*Vd2LysM*) was found in a lineage-specific region of *V. dahliae* strain VdLs17. As it was demonstrated that only Vd2LysM contributes to virulence of *V. dahliae*, the role of Vd2LysM during host colonization was further investigated.

The presence of LysM effector genes in the genomes of fungi with a saprophytic lifestyle suggests that LysM effectors contribute to fungal physiology in other manners than through interfering with host immunity. In **Chapter 5** the hypothesis that LysM effectors play a role in the interaction of fungi with other microbes in the environment was investigated, which could even be relevant for plant pathogenic fungi that encounter other microbes at the site of host infection. To this end, assays were developed that allow assessing the attachment and antagonistic effects of particular bacterial species on fungi by employing the fungus *Trichoderma viride*. Subsequently, these assays were used to assess bacterial attachment and antagonistic activity in the absence or presence of LysM effectors.

In **Chapter6**, the major results described in this thesis are discussed and a perspective on the (potential) roles of LysM effectors in fungi with different lifestyles, including patho-

genic as well as non-pathogenic fungi, is presented.

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Chapter 2

Chitin-binding LysM effectors of fungal plant pathogens play differential roles in virulence

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Kombrink A. (2012). Heterologous production of fungal effectors in *Pichia pastoris*. *Methods in Molecular Biology* **835** 209-217.

Abstract

Fungal cell wall chitin plays an important role in the interaction between fungal plant pathogens and their hosts. Plant receptors recognize chitin fragments as 'non-self' molecules and, consequently, activate immune responses to stop invasion of fungal pathogens. The chitin-binding LysM effector Ecp6 from the fungal tomato pathogen *Cladosporium fulvum* was previously demonstrated to interfere with the activation of chitin-induced immune responses. Homologs of Ecp6 are found in the genomes of many fungal species. Characterization of LysM effectors of *Mycosphaerella graminicola*, *Magnaporthe oryzae* and *Colletotrichum higginsianum* demonstrates that the ability to perturb chitin-induced immunity is conserved among LysM effectors of various fungal pathogens. In addition, two LysM effectors from *M. graminicola* protect fungal hyphae against cell wall hydrolytic plant enzymes, demonstrating that LysM effectors differentially contribute to virulence of fungal plant pathogens.

Introduction

Conserved microbial structures, such as bacterial flagellin and fungal chitin, are exposed in the host plant upon infection by microbes. These molecules, designated microbe-associated molecular patterns (MAMPs), may be recognized by plant hosts as non-self molecules, and consequently trigger immune responses that are aimed at stopping the infection (Nürnberger and Brunner, 2002; Jones and Dangl, 2006). Chitin, an unbranched β-1-4-linked *N*-acetyl-glucosamine (GlcNAc) homopolymer and major constituent of the fungal cell wall, is a well-described MAMP that activates immune responses in plants and animals, leading to MAMP-triggered immunity (MTI) (Boller, 1995; Lee et al., 2008; Shibuya et al., 1993; Felix et al., 1993; Kombrink et al., 2011). The first plant chitin immune receptor was identified in rice (*Oryza sativa*) and designated Chitin Elicitor Binding Protein (OsCEBiP) (Kaku et al., 2006). This receptor-like protein does not contain an intracellular signalling domain, but was suggested to form a complex with the Chitin Elicitor Receptor Kinase 1 (OsCERK1) in the presence of chitin (Shimizu et al., 2010). The Arabidopsis CERK1 homolog, AtCERK1, is essential for chitin signalling, while CEBiP homologues do not appear to play a role in this process (Miya et al., 2007, Wan et al., 2008; Shinya et al., 2012; Wan et al., 2012). In addition, LysM-containing membrane receptors were identified in rice (OsLYP4 and OsLYP6) and Arabidopsis (LYK4) that are involved in resistance against fungal and bacterial pathogens (Petutschnig et al., 2010; Liu 2012; Wan 2012).

The fungal pathogen *Cladosporium fulvum* causes tomato leaf mold and has been used as a model to study plant-pathogen interactions (Thomma et al., 2005; Joosten and de Wit, 1999). In a compatible interaction, *C. fulvum* colonizes the apoplastic space of tomato leaves where it secretes effector proteins to establish infection. Various *C. fulvum* effector proteins have been identified, including the Avr4 protein that binds chitin through an

invertebrate chitin-binding domain and localizes at the fungal cell wall to protect fungal hyphae from degradation by plant chitinases (van den Burg et al., 2003, 2004, 2006; van Esse et al., 2007). Another *C. fulvum* effector that is abundantly secreted into the apoplast is extracellular protein 6 (Ecp6), which was shown to be a virulence factor in infection studies using *Ecp6* RNAi strains (Bolton et al., 2008). Ecp6 contains three LysM domains, which suggests chitin-binding ability. Indeed, glycan array analysis and pull down experiments with insoluble carbohydrates revealed that Ecp6 specifically binds to chitin (de Jonge et al., 2010). However, unlike Avr4, the chitin-binding ability of Ecp6 appeared not to play a role in protecting fungal hyphae from degradation by plant chitinases (de Jonge et al., 2010).

Through its chitin binding ability, Ecp6 was hypothesized to affect chitin perception by the host, and in this manner contribute to fungal virulence (de Jonge and Thomma, 2009). The effect of Ecp6 on the induction of chitin-triggered immune responses was measured in various experiments. First, medium alkalinisation of a tomato cell culture, triggered by recognition of chitin hexamer oligosaccharides ($(GlcNAc)_6$), was significantly reduced upon pre-incubation of $(GlcNAc)_6$ with Ecp6. Consistent with this result, addition of Ecp6 also abolished the production of reactive oxygen species (ROS) triggered by chitin (de Jonge et al., 2010). Avr4 is not able to prevent the activation of chitin-induced immune responses in these assays. These observations suggest that Ecp6 competes with plant receptors for chitin binding. This was confirmed in a competition assay in vitro using rice microsomal fractions that contain the CEBiP chitin receptor. Incubation of these fractions with biotinylated $(GlcNAc)_8$ in the presence and absence of Ecp6 demonstrated that Ecp6 prevented binding of $(GlcNAc)_8$ to CEBiP (de Jonge et al., 2010). In conclusion, the chitin-binding effector Ecp6 suppresses chitin-triggered host immune responses.

LysM effectors such as Ecp6, which comprise secreted proteins with a variable number of LysM domains and no other recognizable domains, are widely distributed in the fungal kingdom (de Jonge and Thomma, 2009). This suggests that sequestration of chitin fragments to prevent chitin-triggered immunity is important for pathogenicity and conserved among fungal pathogens. Furthermore, fungal genomes often contain multiple putative LysM effector genes. In this chapter, we describe the functional characterization of LysM effectors of the pathogenic fungi *Mycosphaerella graminicola*, *Magnaporthe oryzae* and *Colletotrichum higginsianum*, which cause leaf blotch disease of wheat, rice blast disease and anthracnose disease on Brassicaceae, respectively (Marshall et al., 2011, Mentlak et al., 2012; Perfect et al., 1999). In contrast to the biotrophic pathogen *C. fulvum*, these three fungal pathogens display a hemi-biotrophic lifestyle which involves an initial biotrophic and symptomless growth phase, followed by a necrotrophic stage during which extensive tissue decay occurs. During biotrophic growth, *M. graminicola* colonizes the apoplast, while both *Magnaporthe oryzae* and *Colletotrichum higginsianum* grow within host cells while being separated from the host cytoplasm by an intact invaginated plant plasma membrane (Kema et al., 1996; Perfect et al., 1999; Wilson and Talbot, 2009). The results described in

this chapter suggest that suppression of chitin-triggered immunity by LysM effectors plays an important role in the establishment of disease by hemibiotrophic pathogens.

Results

LysM effectors of fungal plant pathogens are expressed during host colonization

The *Magnaporthe oryzae* Secreted LysM Protein 1 (Slp1), which displays high homology to Ecp6, was previously found to be specifically induced during biotrophic growth (Mosquera et al., 2009). Furthermore, the genomes of *Mycosphaerella graminicola* and *Colletotrichum higginsianum* were found to carry putative LysM effector genes encoding varying numbers of LysM domains. To identify LysM effectors that play a role in pathogen virulence, the expression of the LysM effector genes during host colonization was tested. In the *M. graminicola* genome, three LysM effector genes were identified, two of which showed induced expression at four and nine days after inoculation during the biotrophic growth phase of the pathogen (Fig. 1A). A clear reduction in expression of both genes was observed at 14

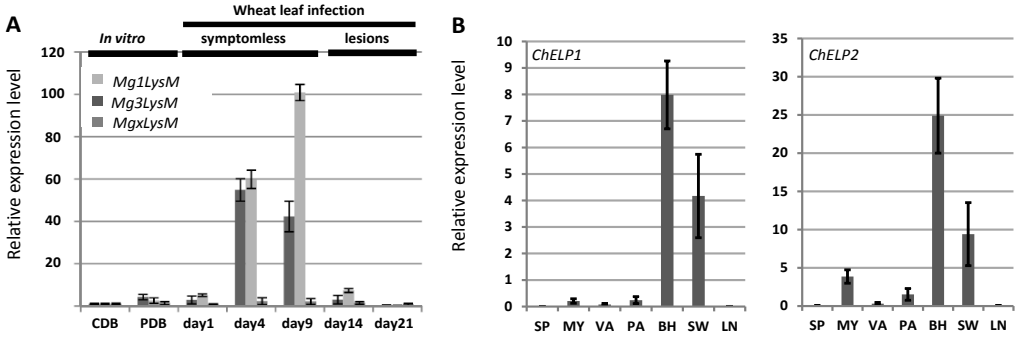

Figure 1. *MgLysM* and *ChELP* genes are expressed in *Mycosphaerella graminicola* and *Colletotrichum higginsianum*, respectively, during plant infection.

A: Real-time RT-PCR expression analysis of *M. graminicola* LysM effector genes during growth in vitro (PDB and CDB) and a time course upon inoculation of wheat plants. The various phases of infection are indicated. Gene expression data are presented relative to the expression of the fungal *β-tubulin* gene. The figure highlights that *Mg1LysM* and *Mg3LysM* are both expressed during symptomless plant infection, whereas *MgxLysM* is not. Graphs are representative of duplicate experiments with similar results.
B: Expression profiling of *C. higginsianum* LysM effector genes assessed using RT-qPCR. Expression levels are shown relative to the mean expression of the two *C. higginsianum* reference genes actin and α-tubulin. In vitro cell types are dormant spores (SP), saprotrophic mycelium (MY) and mature appressoria (VA). *In planta* stages are mature appressoria (PA), early biotrophic phase (BP), biotrophy to necrotrophy switch (SW) and late necrotrophy (LN).

Table I. Summary of LysM effector characteristics

Name	Mature protein length (aa)	No. of LysM domains	No. of Cys residues
CfEcp6	210	3	9
MoSlp1	146	2	6
Mg3LysM	215	3	9
Mg1LysM	79	1	4
ChELP1	152	2	6
ChELP2	158	2	7

days post inoculation when the pathogen switches to necrotrophic growth. The encoded LysM effectors were designated MgxLysM (not expressed), Mg1LysM (with one LysM domain) and Mg3LysM (with three LysM domains). In *C. higginsianum*, expression of seven putative LysM effector genes was observed during infection of Arabidopsis. However, two genes that were most highly expressed, particularly during the biotrophic stage, were selected for further characterization (Fig. 1B). These genes encode LysM effectors with two LysM domains: Extracellular LysM Protein1 (ChELP1) and ChELP2. Characteristics of the LysM effectors are summarized in Table I. An alignment of the LysM effectors shows the sequence similarity among the proteins, which is the lowest at the N-terminus before the start of the first LysM domain while the remainder of the sequences show a high degree of overall similarity (Fig. 2A).

To visualize relationships of the individual LysM domains of the different LysM effectors, a phylogram was generated that includes the three LysM domains of Ecp6 (Fig. 2B). The first LysM domains of the different proteins are clearly more similar to each other than to the second and third LysM domains of the same protein, which suggests that the first domain was present in a common ancestor of the various fungal species. The third LysM domains of Ecp6 and Mg3LysM form a separate cluster within the branch of second LysM domains. This suggests that the third LysM domains of Ecp6 and Mg3LysM might be derived from a duplication of the second domain, which occurred before speciation of *M. graminicola* and *C. fulvum*.

All LysM effectors are chitin binding proteins

LysM effector proteins Mg1LysM, Mg3LysM, Slp1, ChELP1 and ChELP2 were heterologously produced in the yeast *Pichia pastoris* and purified. As *C. fulvum* Ecp6 was shown to specifically bind chitin (de Jonge et al., 2010), the *P. pastoris*-produced LysM effectors were tested for their carbohydrate-binding ability. After incubating with insoluble crab shell chitin, chitosan and plant cell wall-derived xylan and cellulose, all LysM effectors were found to precipitate with chitin and not with any of the other carbohydrates (Fig. 3). For Slp1, multiple bands were visible on Coomassie stained SDS-PAGE gels (Fig. 3). However, only Slp1

A

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Ecp6        1 ----MQSMILFAAALMGAAVNGFVLPRTDD------PDCETKATDCGS-------TSNIK
Mg3LysM     1 -----MQNIFLAATLLGAAFAAPQNPPTSCSAAPQPTGTGSPGTVCGS-------TTFTN
Mg1LysM     1 ----MQFTALVAALLSVAAVQAQRNP----------ITTITPQFDCGA-------TNSQQ
Slp1        1 -MQFATITTLLFAGVAAAMPQATETSAAEP----------SATSTGTP-------GEVVD
ChELP1      1 MQISELFSILAVVGVAAALPQATETTPTPTSASAAATTSATSSPTGSP-------GEVID
ChELP2      1 -MQFSIFTVLAAAASFAVALPVCDATTTATTTSAAANPSPTTSGAANESPTCGKLGDFHK

Ecp6       44 YTVVKGDTLTSIAKKFKS---GIGNIVSVNKLANPNLIELGATLIIPENCSNPDNKSCVS
Mg3LysM    49 YTVKAGDTLGAIAKQYNS---GVCDIAKVNGIDNEDYIKPDQVLSIPANCVTPDNTSCVK
Mg1LysM    39 YVARSGDTLTKIAQEIYHDVVGVCDIARANNLADPNRIDAGTPYTIPINCQTYDRNSCL-
Slp1       43 YTVQCNDTLTIVSQKLNS---GIGNIATLNNLANPNFIALGAVLKVPTAPCVIDNTSCLA
ChELP1     54 YTVVSGDTLTIISQRFSS---GIGNIADASGITNPNFLSLGQVLQVPTYVCTPDNTSCIA
ChELP2     60 TTVKACQTLTTIAERFHS---GIGDIAWQNKLENPNVIFVGQVLLVPVNVCNPDNTSCLV

Ecp6      101 TPAEPHETCVPGLPGSYTIVSGDTLTNISQDFNITLDSLIAANTQIENPDALDVGQTITV
Mg3LysM   106 PVPVITNTCVLGVGSTYTVKSGDSFSAIATSFNITLASLEARNPQIPNYDLIFPGQVINT
Mg1LysM       ------------------------------------------------------------
Slp1      100 KQSD-NNTCVSGWSPYYTIVSGDTFFLVAQKFNLSVDALQAAN-VGADPLLLQLNQVINI
ChELP1    111 KEG--SDTCVEGGPATHTIVAGDTFFLVAQSLGLDVNALLAAN-EGVDPLLLQIGDVINI
ChELP2    117 PVG--EATCVTGGPATYTIKSGDTFFAVAQSLGITTDSLTGAN-PGVVPENLQIDQVINV

Ecp6      161 PVCPSSQCEAVGTYNIVAGDLFVDLAATYHTTIGQIKALNNNVNPSKLKVGQQIILEQDC
Mg3LysM   166 PLCPNSVCDSIGTYVIESGDIFYNLAQSNNVTVGQLESLNVNVNVIDIHPGDIIILEHNC
Mg1LysM       ------------------------------------------------------------
Slp1      158 PTCKN-------------------------------------------------------
ChELP1    168 PVC--------------------------------------------------------
ChELP2    174 PVC--------------------------------------------------------

Ecp6      221 KNVTTAVA
Mg3LysM   226 HNITASA-
Mg1LysM       --------
Slp1          --------
ChELP1        --------
ChELP2        --------
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B

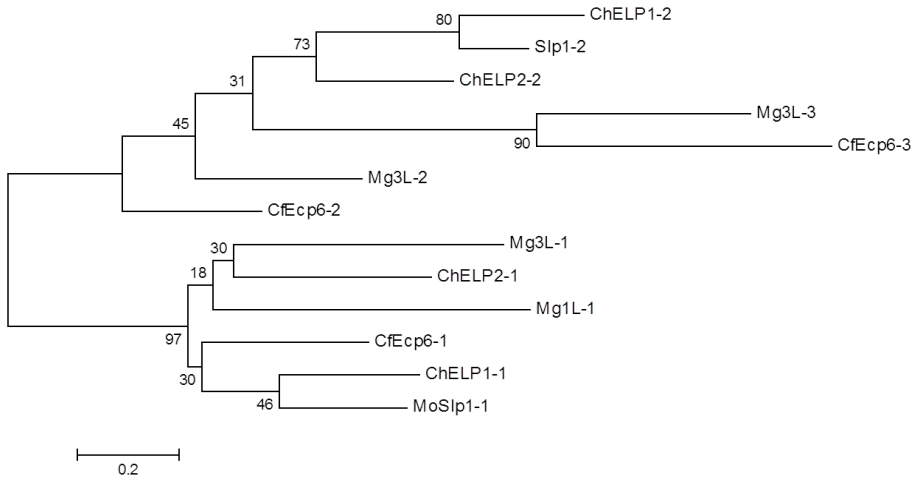

Figure 2. Sequence alignment of LysM effectors and individual LysM domains.
A: Alignment of LysM effectors of *Cladosporium fulvum* (Ecp6), *Mycosphaerella graminicola* (Mg1LysM and Mg3LysM), *Magnaporthe oryzae* (Slp1), and *Colletotrichum higginsianum* (ChELP1 and ChELP2). The LysM domains are underlined.
B: A phylogram of the individual LysM domains of the LysM effectors shown in panel A.

was detected in the gel fragments of the different protein bands with mass-spectrometry, confirming the purity of the protein sample and suggesting that Slp1 forms protein multimers (data not shown). The precipitation results indicate that that chitin-binding ability is conserved among the LysM effectors.

Slp1, Mg3LysM and ChELP1 suppress the activation of chitin-induced plant immune responses

As all Ecp6 homologues bind chitin, we subsequently investigated whether the LysM effectors are functional homologues of Ecp6, and are thus able to suppress chitin-induced immune responses. Therefore, we tested their ability to suppress chitin-induced alkaliniza-

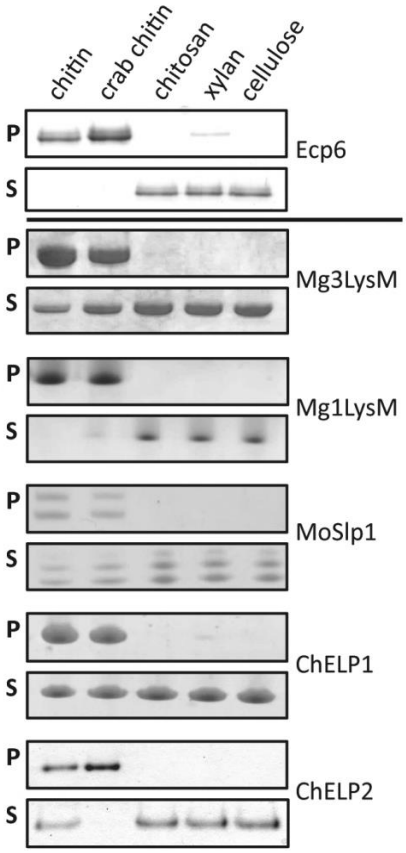

Figure 3. *Pichia pastoris*-produced LysM effectors specifically bind to chitin. LysM effectors of *Cladosporium fulvum* (Ecp6), *Mycosphaerella graminicola* (Mg1LysM and Mg3LysM), *Magnaporthe oryzae* (Slp1), and *Colletotrichum higginsianum* (ChELP1 and ChELP2), were used in affinity precipitation experiments with insoluble polysaccharides. After SDS-PAGE and Coomassie staining, the proteins are observed in the insoluble pellet fraction (P) or in the supernatant fraction (S). All LysM effectors precipitate with chitin, but not with chitosan, xylan and cellulose.

tion of tomato cell cultures. The presence of a ten-fold molar excess of Slp1, Mg3LysM and ChELP-1 significantly suppressed the rapid alkalinisation of the culture medium upon addition of 1nM or 10 nM of $(GlcNAc)_6$ (Fig. 4). These results are consistent with the previously reported activity of Ecp6 (de Jonge et al., 2010). Interestingly, not all LysM effectors suppressed the chitin-induced pH-shift. The presence of 100 nM Mg1LysM did not affect the induction of immune responses by either 1 nM or 10n nM of $(GlcNAc)_6$ (data not shown), suggesting that Mg1LysM does not interfere with the recognition of chitin by tomato cells. The effect of ChELP2 on chitin-triggered immunity could not be measured in the tomato cell suspension, as the addition of ChELP2 by itself already induced a pH-shift. In conclusion, while all LysM effectors bind chitin, the LysM effectors Slp1, Mg3LysM and ChELP1 suppress chitin-induced immune responses of cultured tomato cells while Mg1LysM fails to suppress this immune response.

M. graminicola LysM effectors protect fungal hyphae against hydrolysis

Previously, the chitin-binding protein Avr4 was shown to protect hyphae of *T. viride* against plant hydrolytic enzymes, while incubation of germinating hyphae with Ecp6 did not stop fungal cell lysis (van den Burg et al., 2006; van Esse et al., 2007; de Jonge et al., 2010). We investigated the ability of the LysM effectors to protect fungal hyphae against chitinase ac-

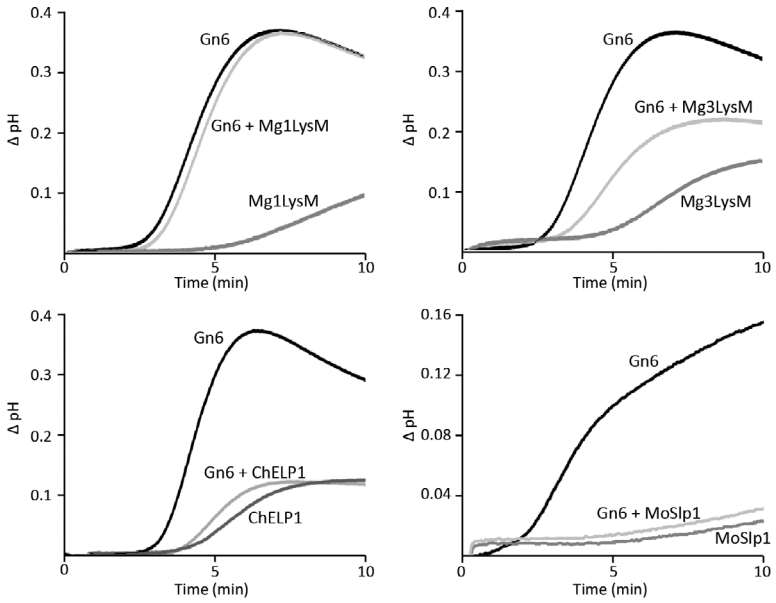

Figure 4. MoSlp1, Mg3LysM and ChELP1, but not Mg1LysM, suppress chitin-induced alkalinisation of a tomato cell suspension. pH-shift measurements in a tomato cell culture after addition of 10 nM chitin (Gn6), 100 nM LysM effector or the combination of both. Graphs represent a representative experiment out of two to four experiments.

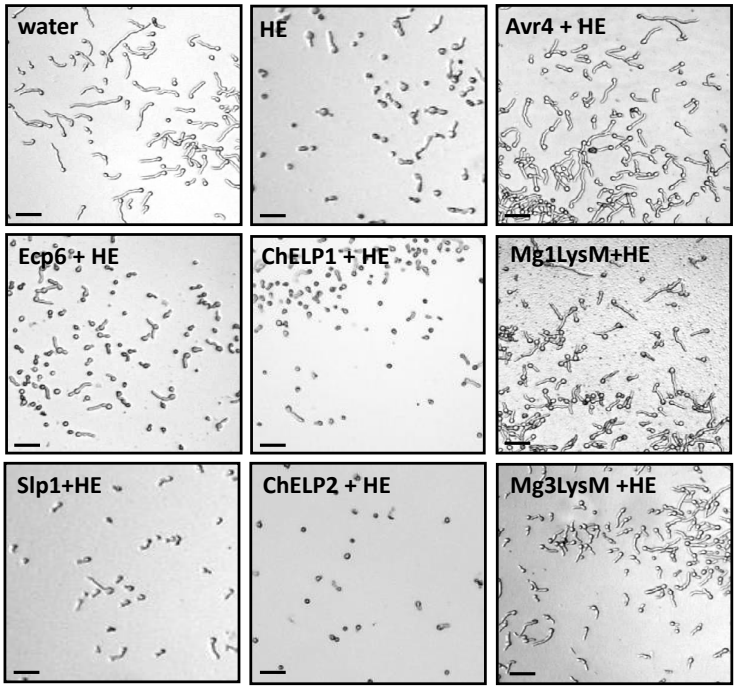

Figure 5. Mg1LysM and Mg3LysM protect fungal hyphae against degradation by hydrolytic enzymes. Pictures of *Trichoderma viride* were taken at 3 to 6 hours after addition of water or a crude tomato plant extract containing hydrolytic enzymes (HE). Prior to addition of the enzymes, the germinating spores were incubated with 10-30 µM of the LysM effector proteins, Avr4 or water. Bars = 20 µm.

tivity. To this end, *T. viride* conidia were germinated, incubated with the LysM effectors, and subsequently treated with a crude extract of tomato leaves containing hydrolytic enzymes (van den Burg et al., 2006). Neither Slp1 nor the ChELP proteins were able to protect the fungal hyphae against hydrolysis (Fig. 5). Interestingly, similar to Avr4 also Mg1LysM and Mg3LysM protected *T. viride* against hydrolysis (Fig. 5). Thus, in contrast to Slp1, ChELP1 and ChELP2, which behave in a similar fashion as Ecp6, Mg1LysM and Mg3LysM protect fungal hyphae against hydrolytic plant enzymes as described previously for Avr4.

Discussion

After the identification of Ecp6 from tomato leaf mold pathogen *Cladosporium fulvum*, putative LysM effector genes were found to be widely distributed in the fungal kingdom (Bolton et al., 2008; de Jonge and Thomma, 2009). In this chapter, we describe the characterization of LysM effectors produced by three additional fungal plant pathogens: *Mycosphaerella graminicola* (Mg1LysM and Mg3LysM), *Magnaporthe oryzae* (Slp1) and *Colletotrichum hig-*

ginsianum (ChELP1 and ChELP2). Interestingly, the results of the in vitro experiments suggest that LysM effectors not only contribute to pathogen virulence through suppression of chitin-induced immune responses as previously described for Ecp6, but also through protection of hyphae against deleterious plant hydrolytic enzymes.

It was previously demonstrated that Ecp6 interferes with chitin-induced host immune responses and that Ecp6 is required for full virulence of *C. fulvum* on tomato. Here, we show that the LysM effectors Mg3LysM, Slp1 and ChELP1 suppress chitin-induced medium alkalinization in a tomato cell suspension in a similar fashion as Ecp6, which suggests that these LysM effectors contribute to virulence of the respective pathogens on their hosts as well. Indeed, *Mg3LysM* and *Slp1* knock-out strains as well as *ChELP*-silencing mutants were severely reduced in virulence on wheat, rice and Arabidopsis respectively, when compared with the respective wild-type strains (Fig. 6) (Marshall et al., 2011; Mentlak et al., 2011). In addition, stronger and more rapid expression of genes involved in MTI was observed during wheat infection of the ΔMg3LysM strain when compared to their expression during colonization by wild-type *M. graminicola* (Marshall et al., 2011). Together with the ability to suppress medium alkalinisation in vitro, these observations indicate that host colonization by these fungal pathogens is facilitated through LysM effector-mediated suppression of chitin-induced immune responses that would otherwise limit fungal growth. Whereas Ecp6 is secreted by the biotrophic fungus *C. fulvum*, the LysM effectors described in this chapter are secreted by hemi-biotrophs that have an initial biotrophic phase after which they switch to necrotrophy. Although hemi-biotrophic pathogens eventually feed and reproduce on dead host tissue, our results suggest that suppression of chitin-triggered host immune responses is essential in the initial stages of infection. However, as LysM effectors even occur in necrotrophic pathogens (de Jonge and Thomma, 2009), suppression of chitin-triggered immune responses might be a conserved mechanism for fungal plant pathogens with diverse lifestyles to overcome host immunity in the first stages of infection.

It was previously suggested that Ecp6 functions through sequestering chitin fragments that would otherwise be perceived by host chitin receptors and trigger immune responses (de Jonge et al., 2010). As the chitin receptor in tomato has not yet been identified, the rice chitin receptor CEBiP was used to demonstrate that Ecp6 has the ability to compete with a plant chitin receptor *in vitro* (Kaku et al., 2006; de Jonge et al., 2010). After the identification of Slp1 from *M. oryzae*, the ability of this rice pathogen LysM effector to compete with rice receptor CEBiP for chitin was observed in a similar assay (Mentlak et al., 2011). Moreover, transgenic rice in which *CEBiP* was silenced by RNAi showed comparable susceptibility upon inoculation with wild-type *M. oryzae* and the *ΔSlp1* mutant strain (Mentlak et al., 2011). These data show that Slp1 is only functional in plants with intact chitin-triggered immunity, and therefore it can be concluded that the virulence function of Slp1 is determined by its ability to perturb CEBiP-mediated chitin-induced immune signalling. Similarly, wheat plants that were silenced for either *CEBiP* or *CERK1* homologues showed equal suscepti-

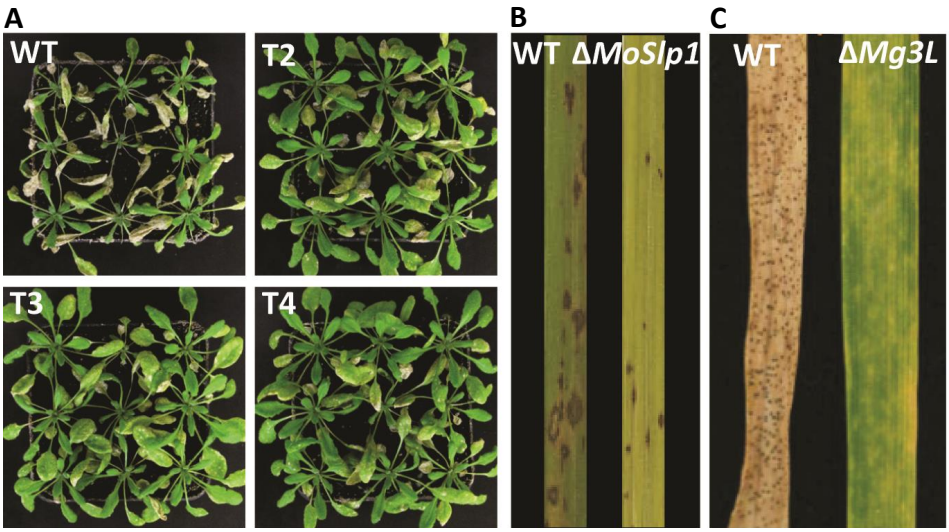

Figure 6. LysM effectors are important for pathogen virulence.

A: Disease symptoms on *Arabidopsis thaliana* Col-0 leaves inoculated either with a *Colletotrichum higginsianum* wild-type strain or three mutants silenced for the expression of *ChELP1* and *ChELP2* (T2, T3, T4). Pictures were taken at 6 days post-inoculation (dpi) (Figure provided by Richard O'Connell, unpublished data).

B: *Magnaporthe oryzae* Guy11 (wild-type [WT]) or Δ*Slp1* mutants were used to inoculate 21-day-old seedlings of the blast-susceptible rice cultivar CO-39. Disease symptoms were reduced on plants inoculated with Δ*Slp1* mutants (reproduced from Mentlak et al., 2012).

C: Leafs infected with wild-type *Mysophaerella graminicola* and a Δ*Mg3LysM* mutant at 30 dpi (reproduced from Marshall et al., 2011).

bility to wild-type and *ΔMg3LysM M. graminicola*. Thus, Mg3LysM interferes with chitin-induced immunity that is mediated by wheat CEBiP and CERK1, which presumably form a signalling complex as previously described in rice (Shimizu et al., 2010; Lee et al., 2013). It is expected that *C. higginsianum* ChELP1 similarly interferes with chitin-induced signalling by Arabidopsis receptors. However, despite the characterization of Arabidopsis chitin receptors, direct experimental proof *in planta* is lacking thus far, which may be due to redundancy among the receptor-like kinases AtCERK1 and AtLYK4 that were both demonstrated to be involved in chitin recognition (Miya et al., 2007; Wan et al., 2008; Wan et al., 2012). Finally, barley contains a receptor with homology to rice CEBiP that was shown to play a role in resistance against *M. oryzae* (Tanaka et al., 2010). As the presence of LysM-domain containing receptors involved in chitin-triggered immunity seems to be widely conserved among plant species, it is likely that many pathogens use LysM effectors to interfere with chitin recognition by receptors of their hosts.

In contrast to the ability of Ecp6, Slp1, ChELP1 and Mg3LysM to suppress the chitin-

induced pH-shift in a tomato cell suspension, Mg1LysM is not able to do so. Thus, despite the ability of Mg1LysM to bind chitin, it presumably does not interfere with chitin-induced host immune responses. Interestingly, both Mg1LysM and Mg3LysM display a novel function, as these proteins have the capacity to protect fungal hyphae against hydrolysis by plant enzymes. This function was previously described for *C. fulvum* effector Avr4 (van Esse et al., 2007). Specifically for Mg3LysM, the ability to protect fungal hyphae indicates that this LysM effector plays a dual role during *M. graminicola* wheat infection. Furthermore, the redundancy between Mg1LysM and Mg3LysM might explain why the ΔMg1LysM mutant strain did not show reduced virulence on wheat. Further research, which includes the generation of a double knock-out mutant, may indicate whether Mg1LysM plays a role in fungal virulence.

LysM effectors consist of LysM domains with short border sequences of 5-28 amino acids and do not contain additional domains. As shown in figure 2B, the first LysM domains of all LysM effectors are more similar to each other rather than to the additional LysM domain(s) within the same protein, irrespective of the phylogenetic relationship of the fungal species that secrete these effectors; *M. graminicola* and *C. fulvum* belong to the class of Dothideomycetes and *C. higginsianum* and *M. oryzae* to the class of Sordariomycetes. Therefore, it is likely that a LysM effector with two LysM domains was present in a common ancestor. Mg1LysM might have lost this second LysM domain or this protein arose from a duplication of the first LysM domain of Mg3LysM. The third LysM domain of Mg3LysM and Ecp6 might be the result of a duplication of the second LysM domain within the class of Dothideomycetes. Unexpectedly, these proteins with three LysM domains that are highly similar differ in their ability to protect hyphae against chitinases. Structural analysis of Ecp6 and Mg3LysM may provide more insight in the contribution of individual LysM domains to their biological activity.

Materials and Methods

Plant and fungal material and handling

Mycosphaerella graminicola

Fungal spores for plant inoculation were harvested from 7-day-old cultures growing (budding) on yeast extract peptone dextrose (YEPD) plates (Oxoid Ltd., Hampshire, UK) at 15°C. A modified version of the fully sequenced *M. graminicola* isolate IPO323 was used (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), which carries a deletion of the *Ku70* gene and increases the rate of generating homologous recombinants via transformation to >70 % (Bowler et al., 2010). For estimation of gene expression *in vitro*, Ku70 was inoculated into cultures of Potato Dextrose Broth (PDB) and Czapek-Dox Broth and shake flask cultures were harvested during logarithmic growth.

For plant infection the second leaf of 17-day-old wheat seedlings of cultivar Riband (susceptible) were

attached, adaxial side up, to Perspex sheets using double sided tape. The inoculation procedure was as described previously (Keon et al., 2007). The leaves were inoculated evenly with fungal spores at a density of 1 x 10^7 cells / ml (or serial dilutions of) in water containing 0.1 % v/v Tween 20. Following 72 hour incubation at 100% relative humidity, inoculated plants were incubated at 16ºC with a 16 hr light period at 88% relative humidity for up to 30 days.

Colletotrichum higginsianum

The genome-sequenced *C. higginsianum* isolate IMI349063A (O´Connell et al., 2012) was used to inoculate Arabidopsis leaves as previously described (Takahara et al., 2009). The fungal developmental stages analysed using RT-qPCR correspond to three *in vitro* stages and four time points in planta during Arabidopsis infection, as previously reported (Kleemann et al., 2012). Saprotrophic mycelium was grown in Mathur´s liquid medium for 5 days.

Gene expression studies

M. graminicola: RNA isolation and real-time RT-PCR

Total RNA was isolated from freeze-dried, filtered fungal material collected during the logarithmic growth phase in liquid cultures or from leaf tissues infected by *M. graminicola* WT (Ku70) using the TRIZOL procedure (Invitrogen). Total RNA was used for all Real-Time RT-PCR analyses. For RT-PCR analysis, first-strand cDNA was synthesized from total RNA using the SuperScript III First_Strand Synthesis System for RT-PCR (Invitrogen). A 5 µg aliquot of total RNA primed with oligo$(dT)_{20}$ was used in a 20 µl reaction, following the suppliers instructions. The resulting cDNA was analysed using a QuantiTect SYBR Green PCR Kit (QIAGEN), following the supplier's instruction. A 0.5 µl aliquot of cDNA was used in a 20 µl PCR reaction, with an annealing temperature of 60°C. Appropriate primers (Supplementary Table 1) were added at a final concentration of 0.25 µM. The PCR reactions were run and analysed using an ABI 7500 Real Time PCR System. For expression normalization the respective beta-tubulin genes were used for *M. graminicola* and wheat.

C. higginsianum: RNAseq

Fungal developmental stages, library preparation and data processing were previously described (O´Connell et al., 2012). To derive the expression patterns of *ChELP* genes, relative expression indexes were calculated as the ratio between the normalised number of reads detected for a given gene at a given fungal developmental stage and the geometrical mean number of reads calculated across the four developmental stages (Duplessis et al., 2011).

Phylogram construction

LysM domain sequences were aligned using CLUSTALW within MEGA4. The obtained alignment was used as input to generate the phylogenetic tree with the maximum likelihood algorithm using the Whalen and Goldman model (WAG) and alignment gaps were removed using the complete deletion option. Boot-

strap values were obtained using 100 sampling replicates.

Heterologous expression in *Pichia pastoris*

All genes were cloned into *P. pastoris* expression vector pPic9 (Invitrogen) after performing PCR using primers to add the N-terminal HIS- and FLAG-tag and *Eco*RI and *Not*I restriction sites for directional cloning (Supplementary Table 1). Subsequently, *P. pastoris* strain GS115 was transformed and a selected clone was cultured in a fermentor (Bioflo 3000) as described previously (Rooney et al., 2005). After removal of cells and concentration of the culture medium the HIS-tagged protein was purified using a Ni-NTA column (Qiagen) according to the manufacturer's protocol. The final protein concentration was determined spectrophotometrically at 280 nm and confirmed using the Pierce BCA Protein Assay Kit (Thermo Scientific, U.S.A.) with bovine serum albumin (BSA) as a standard.

Insoluble carbohydrate binding assays on the LysM proteins

These assays were performed as described (van den Burg et al., 2006) with 50 µg/ml Slp1, 50 µg/ml of Mg3LysM, 600 µg/ml of Mg1LysM and 200 µg/ml of ChELPs. The proteins were incubated overnight at 4ºC or at RT for 1 hr with 3 mg of insoluble polysaccharides while gently rocking.

Chitin induced medium alkalinization assays

These assays were performed as described previously (de Jonge et al., 2010).

Chitinase inhibition assay

A crude extract of tomato leaves containing chitinases was prepared as described previously (Joosten et al. 1995). 40 µL of *Trichoderma viride* spores (104 conidia mL-1) were allowed to germinate overnight, and then incubated with either of the purified LysM effectors (20-30 µM). After 2 hr, crude chitinase extract (5 µl) was added and spores were visualized 4 h later. The recombinant proteins Avr4 and Ecp6 were used as positive and negative controls, respectively (van den Burg et al. 2006, de Jonge et al., 2010).

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Supplemental data

Supplemental Table 1. Primers used in this study

Primer name	Primer sequence 5'-3'	Used for
1LysMcDNAF	ATGCAGTTCACCGCCCTC	Gene expression / cDNA full length cloning of *Mg1LysM*
1LysMcDNAR	TTAGAGGCAGCTGTTGCG	Gene expression / cDNA full length cloning of *Mg1LysM*
3LysMcDNAF	ATGCAGAACATCTTCCTCG	Gene expression / cDNA full length cloning of *Mg3LysM*
3LysMcDNAR	TTAGGCAGACGCAGTGAT	Gene expression / cDNA full length cloning of *Mg3LyM*
MgTubF	ATCACCAGCCCGCAAAGCTT	Fungal gene expression normalisation
MgTubR	ACGATCTTGTGTCCGAGTACCAGC	Fungal gene expression normalisation
Mg1LysMprotF	CGGTATGAATTCCATCATCATCATCATCATCCCGACTACAAGGACGACGA TGACAAGCAGCGGAATCCAATCACCATC	Mg1LysM protein expression
Mg1LysMprotR	CGTCTAGCGGCCGCTTAGAGGCAGCTGTTGCGG	Mg1LysM protein expression
Mg3LysMprotF	CGGTATGAATTCCATCATCATCATCATCATCCCGACTACAAGGACGACGA TGACAAGGCTCCACAAAACCCTCCAAC	Mg3LysM protein expression
Mg3LysMprotR	CGTCTAGCGGCCGCTTAGGCAGACGCAGTGATGTTG	Mg3LysM protein expression
Slp1protF	CGGTATGAATTCCATCATCATCATCATCATCCCGACTACAAGGACGACGA TGACAAGGCCATGCCTCAGGCAAC	Slp1 protein expression
Slp1protR	CGTCTAGCGGCCGCCTAGTTCTTGCAGATGGGGATG	Slp1 protein expression
ChELP1protF	CGGTATGAATTCCATCATCATCATCATCATCCCGACTACAAGGACGAC GATGACAAGCTCCCTCAGGCTACCCC	ChELP1 protein expression
ChELP1protR	CGTCTAGCGGCCGCTTAACAGACGGGGATGTTGATGAC	ChELP1 protein expression
ChELP2protF	CGGTATGAATTCCATCATCATCATCATCATCCCGACTACAAGGACGAC GATGACAAGCTCCCTGTCTGTGACGCC	ChELP2 protein expression
ChELP2protR	CGTCTAGCGGCCGCCTAACACACAGGGACGTTGATG	ChELP2 protein expression

Chapter 3

Two of the three LysM domains of effector Mg3LysM mediate protection of hyphae against plant hydrolytic enzymes

Abstract

LysM effectors from various fungal plant pathogens were demonstrated to bind chitin and to be required for pathogen virulence. Two previously characterized LysM effectors that share a high degree of structural and sequence homology are Ecp6 from tomato leaf mould pathogen *Cladosporium fulvum* and Mg3LysM from wheat pathogen *Mycosphaerella graminicola*. Both Ecp6 and Mg3LysM contribute to virulence of the respective pathogens through interfering with the activation of chitin-triggered immunity. However, Mg3LysM furthermore protects fungal hyphae from degradation by plant hydrolytic enzymes, whereas Ecp6 does not have this ability. Functional analysis revealed that protection against hydrolytic activity from plant enzymes is mediated by the concerted activity of LysM1 and LysM3 in Mg3LysM.

Introduction

The fungal cell wall maintains structural properties of hyphae during growth and morphogenesis, and plays an important role in the interaction of fungi with their environment. Although the composition of the fungal cell wall varies between species and depends on growth conditions and morphological structures even within strains, it basically consists of glycoproteins and polysaccharides, including glucans and chitin (Bowman and Free, 2006; Latgé, 2007). Chitin is a homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) that is essential for the integrity of the fungal cell wall. Furthermore, chitin is known as an important factor in the interaction between fungal pathogens and their plant hosts (Kombrink et al., 2011). As plants do not produce chitin, they evolved to recognize fungal chitin as a non-self molecule by plasma membrane receptors that can activate host immune responses to stop fungal growth (Kaku et al., 2006; Kombrink et al., 2011). These chitin receptors contain extracellular Lysin motifs (LysMs) that are required for their chitin-binding ability. LysM domains occur in prokaryotic and eukaryotic proteins that bind polysaccharides such as chitin and peptidoglycan, a bacterial cell wall-derived chitin-related glycan consisting of *N*-acetylmuramic acid and GlcNAc (Buist et al., 2008). LysM domains consist of approximately 40 amino-acids that adopt a βααβ fold, in which the two α-helices are packed against one side of the double-stranded antiparallel β-sheet. Physical interaction with chitin was demonstrated for several LysM proteins, and occurs in the loop between the first β-strand and the first α-helix and the loop between the second α-helix and β-strand (Ohnuma et al., 2008; Koharudin et al., 2011; Sánchez-Vallet et al., 2013).

To facilitate colonization of their hosts, fungal plant pathogens secrete effectors that perturb host physiology, including host immunity (de Jonge et al., 2011). Several effectors of various fungal pathogens were demonstrated to perturb the activation of chitin-induced immunity: *Cladosporium fulvum* Ecp6, *Mycosphaerella graminicola* Mg3LysM and

Magnaporthe oryzae Slp1 (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012). These effectors are referred to as LysM effectors as they contain a varying number of LysM domains and no other recognizable protein domains (de Jonge and Thomma, 2009). Ecp6, Mg3LysM and Slp1 were demonstrated to bind chitin and are required for full virulence of the pathogens that produce them on their respective host plants tomato, wheat and rice (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012). This suggests that suppression of chitin-triggered immune responses is a conserved mechanism for fungal plant pathogens to facilitate host colonization. The recently analysed crystal structure of Ecp6, a LysM effector that contains three LysMs, revealed that LysM1 and LysM3 cooperatively bind chitin with ultra-high (picomolar) affinity, which allows Ecp6 to outcompete immune receptors for chitin binding (Sánchez-Vallet et al., 2013). The global structure of the Arabidopsis chitin elicitor receptor kinase (AtCERK1) was previously demonstrated to bind chitin with µM affinity trough one of its three LysM domains only (Liu et al., 2012). Interestingly, the singular LysM domain of Ecp6 (LysM2) also contains a chitin-binding site and perturbs chitin-induced defense responses, although it binds chitin with significantly lower affinity than the LysM1-LysM3 composite binding site. This indicates that Ecp6 interferes with chitin-induced immune responses through two separate mechanisms (Sánchez-Vallet et al., 2013).

In addition to its function as microbe-associated molecular pattern (MAMP), fungal cell wall chitin is also a target for plant hydrolytic chitinase enzymes that are released during plant host immune responses and that may cause fungal cell lysis (Schlumbaum et al., 1986). Interestingly, in addition to its ability to interfere with chitin recognition by the host, *M. graminicola* Mg3LysM protects fungal hyphae against degradation by hydrolytic enzymes (Marshall et al., 2011). Although both Ecp6 and Mg3LysM contain three LysM domains and show a high overall sequence similarity, Ecp6 does not protect fungal cell walls against these enzymes (de Jonge et al., 2010). To investigate what determines the ability of Mg3LysM to protect hyphae against chitinases, we performed functional analysis of Mg3LysM and Ecp6. Specifically, through generation of chimeric proteins consisting of LysMs of Mg3LysM and Ecp6, we analysed which of the LysM domains are involved in the ability of Mg3LysM to prevent degradation of fungal hyphae.

Results

Chitin-free Ecp6 does not protect hyphae against hydrolytic enzymes

We previously showed that Ecp6 does not have the capacity to protect fungal hyphae against hydrolysis by plant hydrolytic enzymes by making use of Ecp6 protein that was produced in the heterologous expression host *Pichia pastoris* (de Jonge et al., 2010). Recently, however, a crystal structure of Ecp6 was obtained by making use of the same *P.*

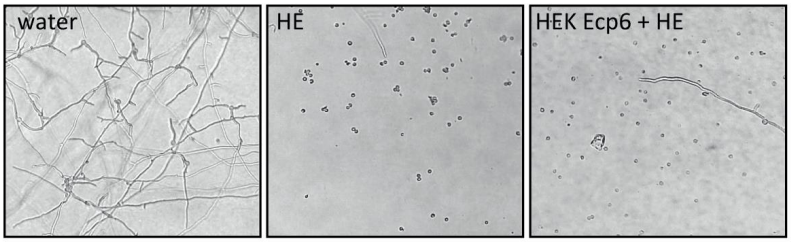

Figure 1. Ecp6 produced in mammalian cells does not protect hyphae against hydrolytic enzymes. Pictures of hyphae of *Trichoderma viride* grown in vitro were taken approximately 20 hours after addition of water or a crude tomato plant extract containing hydrolytic enzymes (HE). Prior to addition of the enzymes, the germinating spores were incubated in 20 µM of Ecp6 or water.

pastoris-produced protein, which remarkably revealed the presence of chitin in the ultra-high affinity binding groove between LysM1 and LysM3, likely derived from *P. pastoris* during heterologous protein production (Sánchez-Vallet et al., 2013). To rule out that the inability of Ecp6 to confer protection against hydrolytic enzymes was caused by chitin that already occupied potential binding sites within the effector protein, we tested the ability of chitin-free Ecp6 produced in mammalian (HEK293) cells to protect hyphae of *Trichoderma viride* against tomato hydrolytic enzymes. As incubation of germinating spores with HEK293-produced Ecp6 protein did not prevent degradation of hyphae by these enzymes (Fig. 1A), it is confirmed that Ecp6 does not have the capacity to protect fungal hyphae against hydrolysis by plant hydrolytic enzymes.

Production of chimeras of Mg3LysM and Ecp6

Although only Mg3LysM was demonstrated to protect fungal hyphae against hydrolytic plant enzymes, Mg3LysM and Ecp6 share a relatively high amino acid sequence identity (47.4 %) (de Jonge et al., 2010; Marshall et al., 2011). However, when compared with Ecp6, the mature peptide of Mg3LysM has an extended N-terminal end. Previously, the Ecp6 mature peptide sequence was experimentally determined through mass spectrometry on protein that was obtained from *C. fulvum* infected tomato (Bolton et al., 2008), whereas the Mg3LysM mature peptide sequence is determined by deleting the signal peptide sequence as predicted by SignalP 4.1 (Fig. 2A) (Petersen et al., 2011). However, it is possible that the signal peptide prediction of Mg3LysM is not correct and, consequently, that the actual mature peptide is shorter. Nevertheless, the N-terminal 18 amino acids of Mg3LysM do not determine its ability to protect fungal hyphae against plant hydrolases, since deletion of these amino acids does not impair this protection (Fig. 2B).

Subsequently, the three dimensional structure of Mg3LysM was predicted using I-TASSER (Zhang, 2008; Roy et al., 2010; Roy et al., 2012), resulting in a structure model with a confidence (C) score of -0.4. Typically, the C-score of a model lies between -5 and 2 and a

model with a C-score higher than -1.5 is generally thought to have a correct fold (Roy et al., 2010). The obtained model indicates that the three-dimensional structure of Mg3LysM is highly similar to that of Ecp6 (Fig. 2C) (Sánchez-Vallet et al., 2013). Therefore, it was anticipated that exchanging LysM domains between Ecp6 and Mg3LysM would not disturb the global arrangement of the LysM domains in the chimeric proteins.

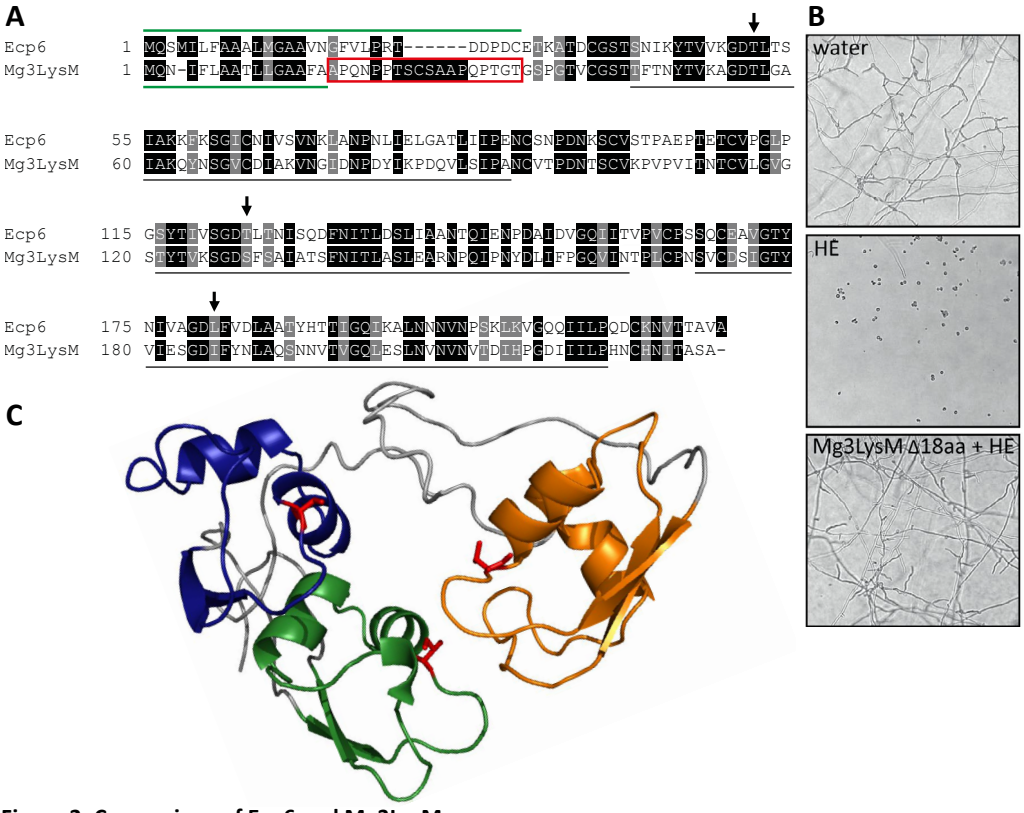

Figure 2. Comparison of Ecp6 and Mg3LysM.

A: ClustalW alignment of Ecp6 and Mg3LysM. The predicted signal peptide of Mg3LysM and the experimentally determined signal peptide of Ecp6 are indicated by the green line. The additional 18 amino acids at the N-terminal mature peptide of Mg3LysM are indicated with the red box. The LysM domains are underlined in black and the arrows indicate the amino acids that were mutated in the three LysM domains.

B: The 18 amino acid extension does not determine the protection ability of Mg3LysM. Pictures of *Trichoderma viride* were taken approximately 20 hours after addition of water or a crude tomato plant extract containing hydrolytic enzymes (HE). Prior to addition of the enzymes, the germinating spores were incubated with Mg3LysMΔ18aa or water.

C: The predicted three-dimensional structure of Mg3LysM as generated with I-TASSER. The residues that were targeted for mutagenesis are shown in red.

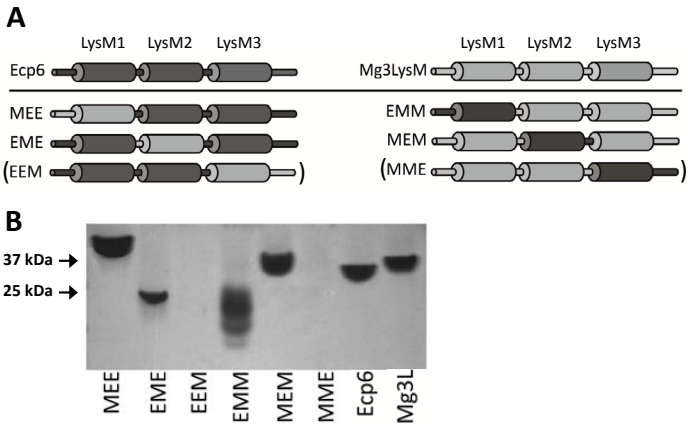

Figure 3. Chimeric proteins of Mg3LysM and Ecp6 bind to chitin.

A: Representation of the chimeric proteins with LysM domains of Mg3LysM and Ecp6. Between brackets are the chimeras that were not obtained.

B: Bis-Tris gel electrophoresis and Coomassie staining of the chimeric proteins, Ecp6 and Mg3LysM.

Constructs encoding affinity-tagged chimeric proteins were generated in which the sequences encoding individual LysM domains of Mg3LysM were swapped, one by one, with the corresponding sequences encoding the corresponding individual LysM domains of Ecp6, and vice versa (Fig. 3A). The HIS-FLAG tagged chimeric proteins were transiently produced in *P. pastoris* and purified using a HIS-column. Unfortunately, SDS-PAGE of the proteins revealed that production of the chimeras in which only the third LysM domain was swapped was unsuccessful in *P. pastoris*, as no protein was obtained after purification of the growth medium of the *P. pastoris* transformants (Fig. 3B). Consequently, these chimeras were not tested in further experiments. Two other chimeras migrated on denaturing protein gel as bands with a lower or higher size than Mg3LysM and Ecp6, whereas the chimera that contains the first LysM domain of Ecp6 combined with the second and third LysM domain of Mg3LysM (EMM) even displayed multiple bands (Fig. 3B). As the predicted sizes of the chimeras lie in between the sizes of Ecp6 and M3LysM, this difference in migration on gel was not expected and might indicate that these chimeras were not produced correctly. Therefore, the proteins were further investigated before testing their ability to protect hyphae against hydrolytic enzymes.

Chitin-binding chimeras suppress chitin-triggered medium alkalinization in a tomato cell suspension

As both Ecp6 and Mg3LysM bind chitin and suppress chitin-triggered immunity (de Jonge et al., 2010; Marshall et al., 2011), it was anticipated that all chimeras would similarly display these characteristics. To investigate their ability to bind chitin, the chimeras were used in an affinity precipitation assay using insoluble crab shell chitin. As all four obtained chimeric proteins were pelleted with chitin, they were demonstrated to bind chitin (Fig. 4A). Subsequently, it was tested whether the chitin-binding chimeras have the ability to suppress chitin-induced immune responses. It was previously demonstrated that tomato cells

respond to addition of chitin oligomers by the activation of immune responses, including alkalinisation of the medium (Felix, 1993). However, incubation of chitohexaose $(GlcNAc)_6$ with Ecp6 or Mg3LysM prior to the addition to the tomato cells significantly suppresses medium alkalinisation, demonstrating that these LysM effectors suppress chitin-triggered immune responses (de Jonge et al. 2010; Marshall et al. 2011). Incubation of chitohexaose with a 10-fold excess of any of the four chimeras similarly suppressed medium alkalinisation (Fig. 4B). Thus, despite the unpredicted migration behaviour of three of the chimeric proteins on gel, all chimeras have the ability to bind chitin and to suppress chitin-induced immunity.

Protection against hydrolytic enzymes is determined by LysM1 and LysM3 of Mg3LysM

To investigate which LysM domain(s) of Mg3LysM is (are) involved in protection against hydrolytic enzymes, we incubated germinating *T. viride* spores in absence or presence of either of the chimeric proteins, Ecp6 or Mg3LysM prior to the addition of hydrolytic en-

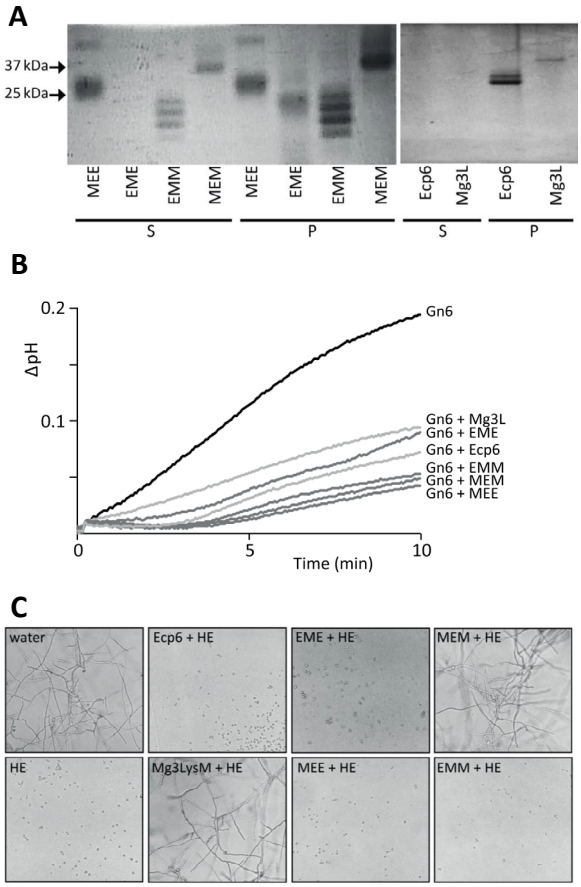

Figure 4. LysM1 and LysM3 of Mg3LysM are required for protection against hydrolytic enzymes.

A: Affinity precipitation of (chimeric) LysM effectors with insoluble chitin. Effector proteins remaining in supernatant (S) and the insoluble chitin pellet (P) after gel electrophoresis and Coomassie staining is shown.

B: The pH-shift was measured after addition of 1 nM of chitin (Gn6), 10 nM of the chimeras, Ecp6, or Mg3LysM or the combination of both. The graphs represent a representative measurement out of two to four measurements in one experiment. The experiment was performed twice with similar results.

C: Pictures of *Trichoderma viride* were taken approximately 20 hours after addition of water or a crude tomato plant extract containing hydrolytic enzymes (HE). Prior to addition of the enzymes, the germinating spores were incubated with 20 µM of the chimeras, Ecp6, Mg3LysM or water.

zymes. Evaluation of the hyphal growth after overnight incubation showed that the chimer containing both LysM1 and LysM3 from Mg3LysM (MEM) protected fungal hyphae, whereas the other chimeras were not able to prevent degradation of the hyphae by plant hydrolytic enzymes (Fig. 4C). However, it should be noted that the MEM chimer is the only protein that migrates on gel as a band of approximately the same size as Ecp6 and Mg3LysM.

Targeted mutagenesis in the LysM domains of Mg3LysM

To further investigate the contribution of the LysM domains of Mg3LysM to the ability of Mg3LysM to protect fungal hyphae, targeted mutation of the individual LysM domains of Mg3LysM was pursued. To this end, three constructs were made with targeted nucleotide substitutions that cause single amino acid changes in the chitin-binding sites of each of the LysM domains of Mg3LysM (Fig. 2A and 2C) (Sánchez-Vallet et al., 2013). These chitin-binding sites are located in the in the loop between the first β-strand and the first α-helix and generally conserved among LysM domains of LysM domain-containing proteins (Buist et al., 2008). The selected amino acids, T^{40}, S^{113}, and I^{170} were substituted by arginine that, due to its size, likely interferes in chitin-binding. Previously, mutations at the same positions were made in the LysM domains of Ecp6 and were found to disrupt their ability to bind chitin (Sánchez-Vallet et al., 2013). Unfortunately, however, heterologous production of the mutants in the second and third LysM domain of Mg3LysM was unsuccessful in *P. pastoris*, as no yield was obtained after purification of the growth medium of the *P. pastoris* transformants. Another attempt to produce the third LysM domain mutant with a substitution to alanine instead of arginine was similarly unsuccessful (Fig. 5A). Nevertheless,

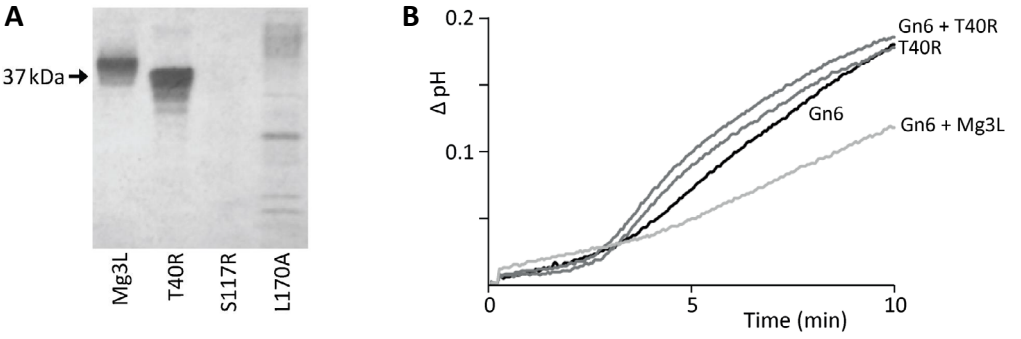

Figure 5. The mutant in LysM1 of Mg3LysM triggers a pH-shift in the absence of chitin

A: Bis-Tris gel electrophoresis and Coomassie staining of the Mg3LysM mutant in LysM1 (T40R), in LysM2 (S117R) and in LysM3 (L170A).

B: pH-shift measurements in a tomato cell suspension after addition of 1 nM chitin or 1 nM chitin that is pre-incubated with 10 nM of the Mg3LysM mutant in LysM1 (T40R). The graphs represent a representative measurement out of two to three measurements in one experiment. The experiment was performed twice with similar results.

the mutant in the first LysM domain of Mg3LysM was obtained (Fig. 5A). Subsequently, this mutant was tested for its ability to suppress chitin-induced medium alkalinisation in a tomato cell suspension. Unfortunately, the LysM1 mutant activated medium alkalinisation already in the absence of chitin, which might indicate that the protein is not folded correctly.

Discussion

In this chapter it was investigated which LysM domain(s) contribute to the ability of Mg3LysM to protect fungal hyphae against plant hydrolytic enzymes. It was observed that the chimeric protein that contains the second LysM domain of Ecp6 (MEM) is able to protect fungal hyphae, which indicates that this ability of Mg3LysM is determined either by LysM1, by LysM3 or by the concerted activity of these two domains. In Ecp6, LysM1 and LysM3 were demonstrated to form a binding groove that interacts with four GlcNAc residues of a chitin oligomer, which is enabled by the long and flexible linker between LysM1 and LysM2 (Sánchez-Vallet et al., 2013). The predicted structure of Mg3LysM suggests that it has a similar fold that would similarly allow the binding of a chitin molecule between LysM1 and LysM3 (Fig. 2C). This is supported by preliminary data from isothermal titration calorimetry (ITC) measurements, which reveal one binding site in *P. pastoris* produced Mg3LysM that binds chitin with low micromolar affinity. This may suggest that the binding-groove formed by LysM1 and LysM3 is occupied by a chitin molecule and only the second LysM domain is available for chitin binding, as was previously observed for *P. pastoris*-produced Ecp6 (A. Sánchez-Vallet, personal communication). It is tempting to speculate that Mg3LysM localizes to the fungal cell wall and, in this manner, protects against host hydrolytic enzymes, whereas Ecp6 does not localize to the fungal cell wall. It could be hypothesized that the amino acid variation that occurs between Ecp6 and Mg3LysM in LysM1 and LysM3 allows binding of Mg3LysM to a (chitin-related) glycan in the fungal cell wall which is not bound by Ecp6, or bound with significantly less affinity. Ligand specificity among homologues LysM proteins has previously been demonstrated for LysM-containing NOD factor receptors in legume plants that perceive lipo-chitooligosaccharide nodulation factors secreted by rhizobium bacteria (Radutoiu et al., 2007). These chitin-related molecules consist of a GlcNAc backbone of four to five residues and are decorated with side groups that allow specificity of the interaction between different rhizobial species and receptors of specific hosts (Radutoiu et al., 2007). An alternative hypothesis for the mechanism by which Mg3LysM protects fungal hyphae is that Mg3LysM, but not Ecp6, has the ability to form a larger complex consisting of multiple Mg3LysM molecules that allows shielding of the fungal hyphae. However, it is difficult to predict whether and how such a complex of Mg3LysM proteins could be established without further knowledge of the Mg3lysM structure.

It was demonstrated previously that the singular LysM2 domain of Ecp6 suppresses chitin-induced medium alkalinisation in a tomato cell suspension (Sánchez-Vallet et al., 2013). Based on the protection against hydrolytic enzymes that is displayed by the MEM chimer, LysM2 of Mg3LysM seems not to be required for the ability to protect fungal hyphae and this domain is most likely involved in the suppression of chitin-triggered immune responses. However, according to the results described in this chapter, it is not possible to discriminate between the involvement of LysM1 and LysM3 of Mg3LysM in protection of fungal hyphae or in the suppression of chitin-induced immunity. In an attempt to investigate the contribution of individual LysM domains to the ability of Mg3LysM to protect fungal hyphae, and in particular to test whether protection is determined by either LysM1 or LysM3, or by their concerted activity, we generated mutants in which the chitin-binding ability of single LysM domains is disrupted. Unfortunately, the mutants in LysM2 and LysM3 were not obtained from *P. pastoris* transformants, whereas Ecp6 mutants with an arginine substitution of amino acids at the same positions in the chitin binding loops were produced (Fig 2A) (Sánchez-Vallet et al., 2013). Possibly, the Mg3LysM mutants in LysM2 and LysM3 were degraded after their production. Although the mutant in LysM1 was obtained, it was not tested for its ability to protect hyphae against hydrolytic enzymes as this mutant triggered an immune response by itself in a tomato cell suspension, suggesting that the protein is not folded correctly. To unravel whether LysM1 or LysM3 or both LysM domains contribute to the ability to protect fungal hyphae, it is crucial to test mutants in the respective LysM domains in the protection assay. Therefore, it might be worthwhile to pursue production of the mutants with an alternative amino acid substitution, or mutants in which amino acids at a different position in the chitin-binding site are substituted, or to produce the mutants in a heterologous expression host that lacks chitin, such as E. coli.

Based on the structural similarity between Ecp6 and Mg3LysM it was expected that swapping of the LysM domains would not disturb the global arrangement of the LysM domains in the chimeric proteins. However, the migration of the chimeric proteins on gel indicates that improper chimeric proteins were produced. MEM is the only chimeric protein that migrates on gel as a band of the expected size (Fig. 3B) and therefore it is not sure whether the other chimeras are correct, despite their ability to bind chitin and suppress chitin-triggered immunity (Fig. 4A,B). After all, it has previously been demonstrated that a singular LysM domain of Ecp6 is sufficient to suppress chitin-triggered immune responses (Sánchez-Vallet et al., 2013). Therefore, the chimer that migrates as a band of a significantly lower size than Ecp6 and Mg3LysM and the chimer that displays multiple bands of a lower size might even represent truncated proteins. Future analysis of the chimeras with mass spectrometry might reveal their composition and allow conclusions on the cause of their unexpected migration on gel. Thus far, it is demonstrated that LysM1 and/or LysM3 are required for the ability to protect fungal hyphae, but more research is needed to elucidate the chitin-binding affinity of these LysM domains and the mechanism by which pro-

tection of hyphae is achieved.

Materials and Methods

Production of chimeric proteins

Primers were designed for overlap extension PCR to construct Ecp6-Mg3LysM chimeras and are presented in Supplemental Table 1. Coding sequences for an N-terminal HIS and FLAG tag were added with PCR, and *Eco*RI and *Not*I endonuclease restriction sites were added for directional cloning. After sequence verification of the chimeric genes in the pGEMT-Easy vector (Promega, Madison, U.S.A.), they were cloned into expression vector pPIC9 (Invitrogen, Carlsbad, U.S.A.) for transformation to *Pichia pastoris* strain GS115. Selected *P. pastoris* transformants were subsequently cultured in a fermentor and the protein was purified from concentrated culture medium using a nickel-nitrilotriacetic acid agarose column (Qiagen, Leusden, the Netherlands). After elution from the column, the proteins were dialysed against demineralized water and the concentration was determined using absorbance at 280 nm.

Protein gels

For Ecp6, Mg3LysM, the chimeric proteins and the targeted mutants in Mg3LysM, a protein sample of approximately 0.5 mg/ml was boiled for 10 minutes in 1x sample buffer containing 5% β-mercaptoethanol and 2% SDS. 20 µl of the sample was loaded on a Bis-Tris buffered acryl-amide gel using SDS-MOPS electrophoresis buffer containing 5mM sodium bisulfite. The gels were stained with Coomassie.

Chitin-binding assay

The produced proteins were tested for their chitin-binding ability as described previously (de Jonge et al., 2010). Briefly, 1 hr after incubation of the purified proteins at room temperature with insoluble crab chitin (Sigma, St. Louis, U.S.A), the suspension was pelleted through centrifugation and a supernatant sample was taken. After three times washing with water, the chitin pellet was boiled in 1% SDS. The presence of the protein in the supernatant and pellet samples was examined with SDS-PAGE and Coomassie Brilliant Blue staining.

Medium alkalinisation assay

The suppression of chitin-induced medium alkalinization in a tomato cell suspension by the produced proteins was tested as described previously (de Jonge et al., 2010), using final concentrations of 1 nM of $GlcNAc_6$ and 10 nM of Ecp6, Mg3LysM, chimeras or Mg3LysM mutant proteins.

Hyphal protection assay

The hyphal protection assays were performed as described previously (de Jonge et al., 2010). Pictures were taken approximately 20 hours after addition of a crude extract of tomato leaves containing hydrolytic enzymes.

Targeted mutagenesis of Mg3LysM

To obtain single amino acid substitutions in the protein sequences, forward and reverse primers were designed for each mutant that contained corresponding nucleotide polymorphisms (Supplemental Table 1). PCR using Pfu polymerase (Promega, Madison, U.S.A.) was performed on *Mg3LysM* in the pGEMT-easy plasmid that already contained coding sequences for the N-terminal HIS and FLAG tags and *Eco*RI and *Not*I sites for cloning in pPIC9. After amplification of the entire plasmid, *Dpn*I (New England Biolabs, Beverly, U.S.A.) digestion was done on the PCR reaction to digest the methylated template plasmid. After on-column cleaning, the pGEMT-easy plasmid with mutagenized *Mg3LysM* was transformed to *E. coli* strain DH5α for amplification. After sequence verification, the *Mg3LysM* mutants were cloned into pPIC9 (Invitrogen, Carlsbad, U.S.A.) for protein production in *P. pastoris*.

Prediction of the three-dimensional structure of Mg3LyM

A model of Mg3LysM was created using the I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Zhang, 2008; Roy et al., 2010; Roy et al., 2012). For this application, the amino acid sequence of Mg3LysM was threaded onto the structure of Ecp6 (PDB accession 4B8V) (Sánchez-Vallet et al., 2013).

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Supplemental data

Supplemental Table 1. Primers used in this study

Primer name	Primer sequence 5'-3'	Used for generation of
Mg3L-F	CGGTATGAATTCCATCATCATCATCATCCCGACTACAAGGACGACGATGACAAGGCTCCACAAAACCCTCCAAC	MEE/MME/MEM
Mg3L-Ecp6-OE-R	CGGGGTTAGAACAGTTCTCGGGAATGCTGAGGACTTGGTC	MEE
Ecp6-Mg3L-OE-F	GACCAAGTCCTCAGCATTCCCGAGAACTGTTCTAACCCCG	MEE
Ecp6-R	CGTCTAGCGGCCGCTTATGCCACAGCAGTAGTGACG	MEE/MME/EME
Ecp6-F	CGGTATGAATT**C**CATCATCATCATCATCATCCCGACTACAAGGACGACGATGACAAGGAAACCAAAGCGACGGACTG	EEM/EME/EMM
Ecp6-Mg3L-OE-R	CGGACTCAATGACATAGGTACCGACAGCCTCGCACTGGGAC	EEM
Mg3L-Ecp6-OE-F	GTCCCAGTGCGAGGCTGTCGGTACCTATGTCATTGAGTCCG	EEM
Mg3L-R	CGTCTAGCGGCCGCTTAGGCAGACGCAGTGATGTTG	EEM/MEM/EMM
Ecp6-Mg3L-OE-R2	CCGGACTTGACGGTGTAGGTGCCTGGAAGACCTGGCACGC	EME
Mg3L-Ecp6-OE-F2	GCGTGCCAGGTCTTCCAGGCACCTACACCGTCAAGTCCGG	EME
Mg3L-Ecp6-OE-R2	CACTGGGACGATGGGCAGACAGGCGTGTTGATGACCTGGC	EME
Ecp6-Mg3L-OE-F2	GCCAGGTCATCAACACGCCTGTCTGCCCATCGTCCCAGTG	EME
Ecp6-Mg3L-OE-R3	TCCGGGGTGACGCAGTTGGCTGGGATGATGAGGGTTGCGC	EMM
Mg3L-Ecp6-OE-F3	GCGCAACCCTCATCATCCCAGCCAACTGCGTCACCCCGGA	EMM
Mg3L-Ecp6-OE-R3	CCGGCCACAATGTTGTAAGTGCCAATGCTGTCGCACACGG	MME
Ecp6-Mg3L-OE-F3	CCGTGTGCGACAGCATTGGCACTTACAACATTGTGGCCGG	MME
Mg3L-Ecp6-OE-R4	CCGCTGACGATGGTGTAGCTAGATCCCACACCCAGCACGC	MEM
Ecp6-Mg3L-OE-F5	GCGTGCTGGGTGTGGGATCTAGCTACACCATCGTCAGCGG	MEM
Ecp6-Mg3L-OE-R4	CACACGGAGTTGGGGCAGAGTGGGACGGTGATGATCTGGC	MEM
Mg3L-Ecp6-OE-F4	GCCAGATCATCACCGTCCCACTCTGCCCCAACTCCGTGTG	MEM
Mg3L-T40R-F	GTGATAGGCTCGGCGCCATCGCCAAGCA	Mg3L-T40R
Mg3L-T40R-R	GCCGAGCCTATCACCGGCCTTGACGGTG	Mg3L-T40R
Mg3L-S117R-F	CCGGCGACAGGTTCTCCGCCATCGCCACCTCGT	Mg3L-S117R
Mg3L-S117R-R	GCGGAGAACCTGTCGCCGGACTTGACGGTGTA	Mg3L-S117R
Mg3L-I170R-F	GGCGACAGGTTCTACAACCTCGCCCAGAG	Mg3LI170R
Mg3L-I170R-R	GTAGAACCTGTCGCCGGACTCAATGACAT	Mg3LI170R

Chapter 4

A lineage-specific LysM effector of *Verticillium dahliae* contributes to virulence

Part of this chapter is published in:

de Jonge R., Bolton M.D.*, Kombrink A.*, van den Berg G.C.M.*, Yadeta K.A., Thomma B.P.H.J. (2013). Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen. *Genome Research* **23** 1271-1282 (*equal contribution).

Abstract

Chitin-binding LysM effectors of various foliar fungal pathogens were previously demonstrated to contribute to virulence. In this chapter, LysM effectors of the soil-borne vascular fungal plant pathogen *Verticillium dahliae* are described. Comparative genomics of eleven *V. dahliae* strains revealed that four LysM effectors are found in the core genome, which are referred to as core VdLysM effectors. In addition to the core genome, *V. dahliae* strains generally carry lineage-specific (LS) genomic regions. Interestingly, an additional VdLsyM effector gene (*Vd2LysM*) occurs in a LS region of *V. dahliae* strain VdLs17 and is absent in all other sequenced *V. dahliae* strains. Whereas a role in virulence could not be attributed to any of the four core LysM effectors, the LS effector Vd2LysM contributes to virulence of strain VdLs17. Vd2LysM binds chitin and suppresses chitin-induced immune responses, which indicates that Vd2LysM interferes with chitin-induced immunity during host colonization by *V. dahliae* strain VdLs17.

Introduction

To establish infection, fungal plant pathogens secrete effector molecules that manipulate host physiology, including immune responses that are triggered when plant hosts sense invading pathogens (Jones and Dangl, 2006; de Jonge et al., 2011). Typically, effectors are small secreted proteins that are species- or even strain-specific. However, some effectors are more broadly conserved, such as the necrosis- and ethylene-inducing-like proteins (NLPs) that are produced by bacteria, oomycetes and fungi, and that are particularly known for their phytotoxic activity (de Jonge et al., 2011; Gijzen and Nürnberger, 2006). Another group of conserved fungal effectors are lysin motif (LysM) effectors which are found in a wide range of fungal species, including plant and animal pathogens and saprophytes (de Jonge and Thomma, 2009).

LysM effectors are defined as secreted proteins that contain no annotated protein domains apart from a varying number of LysM domains, which are carbohydrate binding modules that occur in many prokaryotic and eukaryotic proteins (de Jonge and Thomma 2009; Buist et al., 2008). The LysM effectors studied to date belong to various fungal plant pathogenic species and were all found to bind chitin, a homopolymer of unbranched β-1-4-linked *N*-acetyl-glucosamine (GlcNAc) (de Jonge et al., 2010; Marshall et al., 2011, Mentlak et al., 2012). Chitin is a major component of fungal cell walls and plays an important role in the interaction between fungal pathogens and their plant hosts (Bowman and Free, 2006; Kombrink et al., 2011). Plants evolved to recognize chitin as a 'non-self' molecule and mount an immune response upon chitin perception in order to stop fungal infection (Felix et al., 1993; Shibuya et al., 1993). Several plasma membrane-localized chitin receptors have been identified in plants that all contain LysM domains (Kaku et al., 2006; Kom-

brink et al., 2011). LysM effectors of various fungal species were demonstrated to perturb the activation of chitin-induced immunity during host colonization and contribute to virulence (de Jonge et al., 2010; Marshall et al., 2011, Mentlak et al., 2012). Recently, the crystal structure of the LysM effector Ecp6, secreted by the tomato leaf mould pathogen *Cladosporium fulvum*, revealed that Ecp6 suppresses chitin-triggered immunity through two distinct mechanisms (Sánchez-Vallet et al., 2013). One mechanism involves two LysM domains of a single Ecp6 molecule (LysM1 and -3) that cooperatively bind chitin with ultra-high (pM) affinity and allows Ecp6 to outcompete plant receptors for chitin binding. In addition also the remaining, singular, LysM domain (LysM2) binds chitin and has the capability to suppress chitin-induced immune responses. However, its relatively low (μM) affinity for chitin likely does not allow this domain to function through sequestration of chitin fragments, suggesting that LysM2 suppresses chitin-triggered immunity through another mechanism (Sánchez-Vallet et al., 2013).

In addition to *C. fulvum* Ecp6, LysM effectors of three other fungal plant pathogens were characterized, and were found to interfere with the activation of chitin-induced host immunity: *Mycosphaerella graminicola* Mg3LysM, *Magnaporthe oryzae* Slp1 and *Colletotrichum higginsianum* ChELP1 (Marshall et al., 2011; Mentlak et al., 2012, Chapter 2). Furthermore, LysM effectors Mg1LysM and Mg3LysM from *M. graminicola* were found to protect fungal hyphae against degradation by plant chitinases (Schlumbaum et al., 1986; Marshall et al., 2011). Although the LysM effectors that are characterized to date are all secreted by fungal leaf pathogens, they are also found in the genomes of soil-borne pathogens that infect host plants through their roots, such as *Fusarium oxysporum* and *Verticillium dahliae* (de Jonge and Thomma, 2009).

V. dahliae is a soil-borne fungal pathogen that colonizes the xylem vessels of its host plants, resulting in vascular wilt disease (Fradin and Thomma, 2006; Klosterman et al., 2009). *V. dahliae* infects a wide range of dicotyledonous plant species, including economically important crops such as cotton, lettuce and tomato. Genetic resistance has been characterized in tomato, and it was shown that the cell surface localized immune receptor Ve1 confers resistance against strains of *V. dahliae* that belong to race 1 (Fradin et al., 2009). Based on comparative population genomics it was recently discovered that Ve1 recognizes the race 1-specific effector protein Ave1 (for Avirulence on Ve1 tomato) in order to activate effector-triggered immunity (de Jonge et al., 2012). Furthermore, Ave1 is required for full virulence on tomato genotypes that lack Ve1 (de Jonge et al., 2012). Interestingly, Ave1 homologues occur in various plant pathogenic fungi, although it remains to be demonstrated whether these also act in virulence on their hosts (de Jonge et al., 2012). The comparative population genomics that was performed to identify Ave1 furthermore revealed that all *V. dahliae* strains carry lineage-specific (LS) genomic regions that account for 1-5 Mb of their 32-35 Mb genome (de Jonge et al., 2012; 2013). While in several other plant pathogenic species, such as *Fusarium oxysporum* and *Mycosphaerella graminicola*,

such LS regions are found as small dispensable chromosomes, in *V. dahliae* these regions are found as islands within the core chromosomes (Ma et al., 2010; Goodwin et al., 2011; de Jonge et al., 2013).

In this chapter we describe the LysM effector gene family of *V. dahliae* that consist of four members in the genomes of ten *V. dahliae* strains. Remarkably, a contribution to virulence could not be attributed to any of these four core LysM effector genes. Interestingly, one additional LysM effector gene was found in an LS region of *V. dahliae* strain VdLs17 that was found to play a role during host colonization.

Results

Four core LysM effectors are identified in *Verticillium dahliae*

Previously, LysM effectors were identified in the genome of *V. dahliae* strain VdLs17 (Klosterman et al., 2009). However, gene functional analysis has not been established for this strain, which was isolated from the non-model plant lettuce and is a rather mild pathogen of the model plants tomato and Arabidopsis (Yadeta and Thomma, unpublished data). Therefore, we chose to analyse LysM effectors in *V. dahliae* strain JR2, which is frequently used for gene functional analysis in our laboratory as this strain is a virulent pathogen on these model plants (de Jonge et al., 2012; 2013; Santhanam et al., 2013; Santhanam and Thomma, 2013).

Initially six LysM effector genes that were found in VdLs17 were also found to occur in JR2 (Klosterman et al., 2009; de Jonge et al., 2013). In the present study the corresponding gene models were revisited, revealing that not all of the initially identified effector genes have been predicted correctly. Firstly, in contrast to previously studied LysM effectors of which LysM domains constitute the major portion of the protein, *VDAG_03096* encodes a LysM effector that is predicted to contain a single LysM domain that constitutes only a small portion of the total protein (Fig. 1). Furthermore, mapping of RNA sequencing reads from samples of *V. dahliae* grown *in vitro* and *in planta* (de Jonge et al., 2012; Faino et al., 2012) indicated that the gene prediction of *VDAG_03096* as presented in the database of the Broad Institute is incorrect, as reads mapped to predicted introns while other parts of the predicted gene, including the LysM domain, were not supported by mapped read (Fig 1). Therefore, we did not pursue further characterization of this locus. Similarly, deeper analysis of the predicted protein sequence that corresponds to *VDAG_06426* by SMART (http://smart.embl-heidelberg.de) revealed absence of a signal peptide and presence of a Zinc finger domain (Fig. 1), disqualifying this gene as a bona fide LysM effector gene so it was excluded from further experiments as well. The remaining four LysM effector genes *VDAG_00902*, *VDAG_08171*, *VDAG_04781* and *VDAG_6998* do not only occur in strains VdLs17 and JR2, but also occur in nine additional *V. dahliae* strains that were sequenced

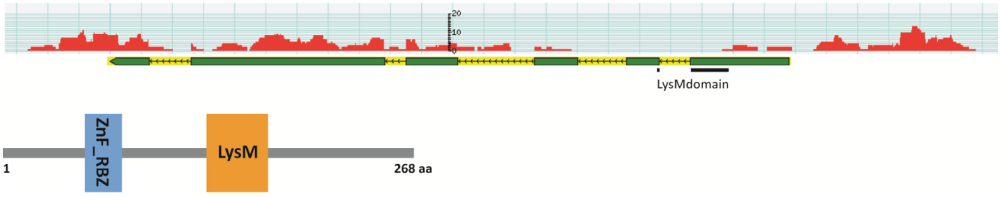

Figure 1. Disqualification of two previously identified *Verticillium dahliae* LysM effector genes.
Two originally identified VdLysM effector genes are not predicted correctly. The initially predicted gene model of LysM effector gene *VDAG_03096* is not supported by mapping of RNA sequencing reads (in red). Reads map to predicted introns (in yellow), whereas some coding parts of the gene (in green), including the LysM domain that constitutes only a small part of the predicted protein, is not supported by reads at all. SMART prediction using the amino acid sequence encoded by *VDAG_06426* reveals absence of a signal peptide and presence of a Zinc finger domain. Also in this case, the LysM domain constitutes only a small portion of the predicted protein.

(de Jonge et al., 2012; 2013) and will be referred to as core VdLysM effector genes. These genes encode proteins with either four or six LysM domains and were named accordingly; *Vd4LysM* (with four LysM domains), *Vd6LysM-1*, *Vd6LysM-2* and *Vd6LysM-3* (with six LysM domains).

VdLysM sequence analysis in eleven *V. dahliae* strains

Evaluation of amino acid sequence similarity among the individual VdLysM domains of the VdLysM effectors of strains VdLs17 and JR2 revealed high (>80%) similarity between LysM domains within individual effectors, suggesting that duplication of LysM domain-encoding sequences occurred within LysM effector genes (Fig. 2A). To investigate allelic variation of VdLysM effector genes, their sequences were derived from the genomes of all of the eleven sequenced *V. dahliae* strains and aligned (Supplemental Table 1). Unexpectedly, some strains were found to contain variants of *Vd4LysM* and *Vd6LysM-3* that are either shorter or longer than the sequences in the genome of strain VdLs17. Strain ST100 contains a short variant of *Vd4LysM* which encodes a protein with three LysM domains that is lacking the second LysM domain that is present in Vd4LysM of VdLs17 (Fig. 2B). An elongated version of Vd4LysM that contains an additional partial LysM domain is encoded in the genome of strain DVD-S26. This partial LysM domain is likely derived from a partial duplication of the third LysM domain, as it is identical to the last 30 of the in total 45 amino acids of that domain. The remaining nine strains contain *Vd4LysM* alleles that are structurally identical to the allele in VdLs17, although two SNPs that are predicted to result in amino acid substitutions were found in strains DVD-3 and DVD-161 (Fig. 2C). For *Vd6LysM-3*, a shorter variant encoding only four LysM domains and that lacks LysM2 and LysM5 was found in

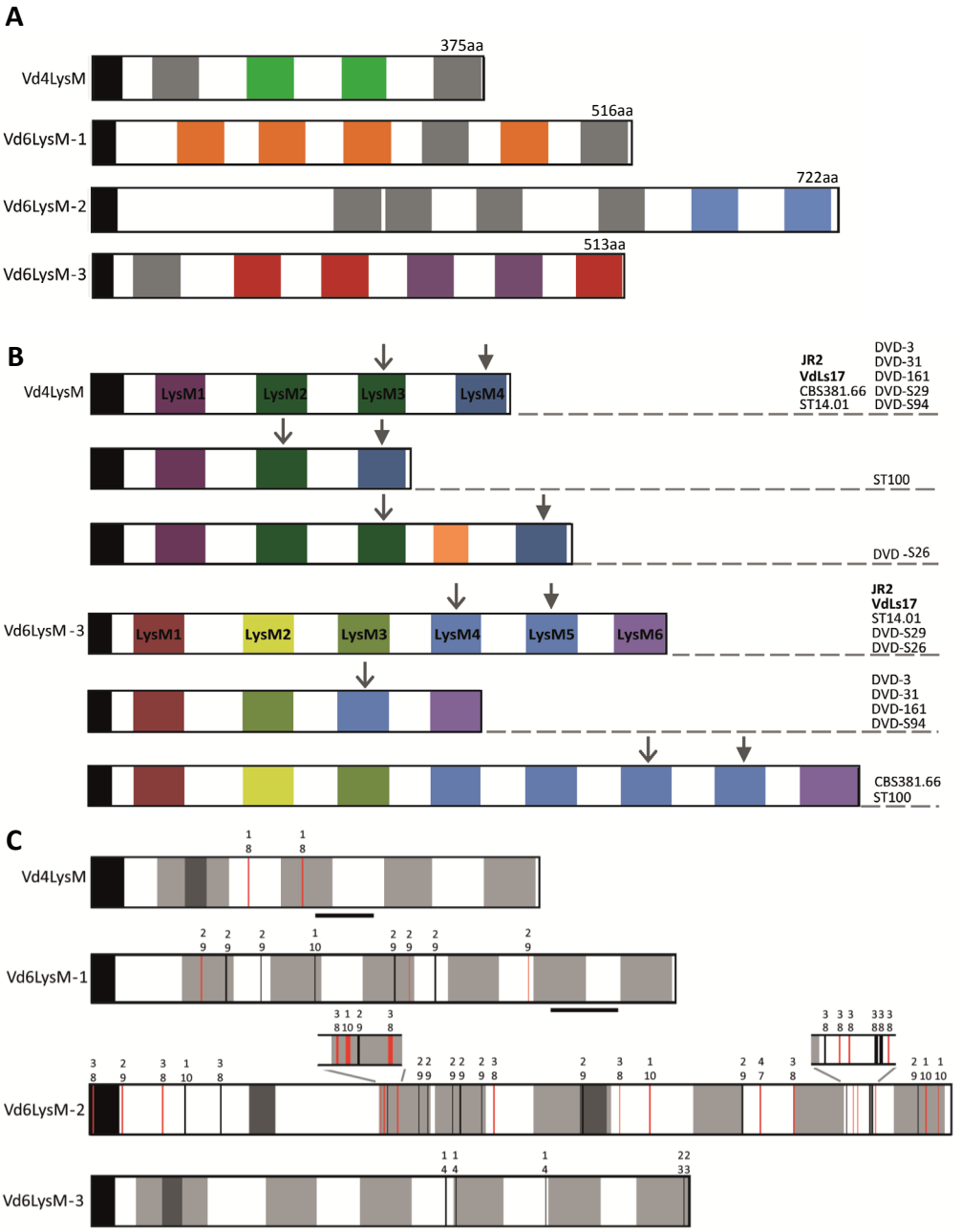

Figure 2. Allelic variation among VdLysM effector genes of eleven *V. dahliae* strains.

A: The four core LysM effectors that are identified in *V. dahliae* have four or six predicted LysM domains that are represented by grey or coloured squares. The N-terminal black boxes represent the predicted signal peptides. Squares of the same colour indicate LysM domains that share >80% identity, except for

the grey squares.
B: Representation of Vd4LysM and Vd6LysM-3 as identified in VdLs17 with below the shorter and longer variants. The signal peptide is indicated as a black box and coloured squares represent LysM domains, of which squares with the same colour represent LysM domains that share >90% identity. Identical LysM domains are indicated by the same (open or closed) arrows.
C: SNP sites in the VdLysM effector genes. The signal peptide is indicated as a black box, introns as dark grey boxes and LysM domains as light grey boxes. Two regions that could not be assessed for the presence of SNPs due to sequencing errors are underlined. Sites with synonymous and non-synonymous nucleotide substitutions are marked by black lines and red lines, respectively. The number of strains that share the same nucleotide polymorphism is indicated above the SNP site.

four *V. dahliae* strains. Two strains, ST100 and CBS381, contain an elongated variant of *Vd6LysM-3* that encodes eight LysM domains (Fig. 2B). In this variant, the fourth and fifth LysM domains are identical to the sixth and seventh LysM domain respectively, suggesting that a duplication of these two LysM domains has occurred. Alignment of the remaining *Vd6LysM-3* alleles found in VdLs17, JR2 and three other strains revealed the presence of five SNP sites (Fig. 2C), three of which consist of synonymous SNPs that occur in strain JR2 only and comprise the only sequence variation that is found between VdLysM effector genes of strains JR2 and VdLs17.

No structural variation is found among alleles of the remaining two core VdLysM effector genes, *Vd6LysM-1* and *Vd6LysM-3*. Only a handful of polymorphic sites were found in *Vd6LysM-1*, while thirty SNP sites were found in *Vd6LysM-3*, sixteen of which encompass amino acid substitutions (Fig. 2C). Most nucleotide substitutions that are identified in *Vd6LysM-1* and *Vd6LysM-2* are simultaneously found in both JR2 and VdLs17, or simultaneously in JR2, VdLs17 and ST100. This is consistent with the phylogeny of the sequenced *V. dahliae* strains that revealed that VdLs17 and JR2 form a separate cluster and that ST100 is phylogenetically separated from all other strains (de Jonge et al., 2013). Although the sequence variation in *Vd6LysM-2* exceeds that of other VdLysM effector genes, it does not indicate that this gene is under purifying or positive selection.

LysM effectors of *V. dahliae* strain JR2 do not contribute to virulence on tomato

To test whether the core LysM effectors contribute to virulence of *V. dahliae* on tomato, gene functional analysis was pursued in *V. dahliae* strain JR2. Previously, RNA-sequencing was performed on samples harvested during a time course of *V. dahliae*-inoculated *N. benthamiana* plants (de Jonge et al., 2012; Faino et al., 2012). These data were used to assess the expression of *VdLysM* genes during this interaction. However, no significant *VdLysM* effector gene expression was observed at any of the time points. In contrast, the expression of the *Ave1* effector gene was significantly induced in these samples (de Jonge

et al., 2013) (Fig. 3A). Differential *V. dahliae* effector gene induction among host plant species has previously been observed, as the NLP2 effector gene was found to be induced in tomato and Arabidopsis, but not in *N. benthamiana* (Santhanam et al., 2013). Therefore, real-time PCR was performed on cDNA generated from samples from tomato plants inoculated with *V. dahliae* strain JR2 and harvested at different time points. However, again no expression of any of the *VdLysM* effector genes was recorded, whereas expression of *Ave1* as a positive control was detected at four, eight and twelve days post inoculation (data not shown). However, it cannot be excluded that *VdLysM* effector gene expression may be induced at low levels only, or only at very specific time points or at particular sites. Therefore, the possible contribution of the core LysM effector genes to virulence of *V. dahliae*

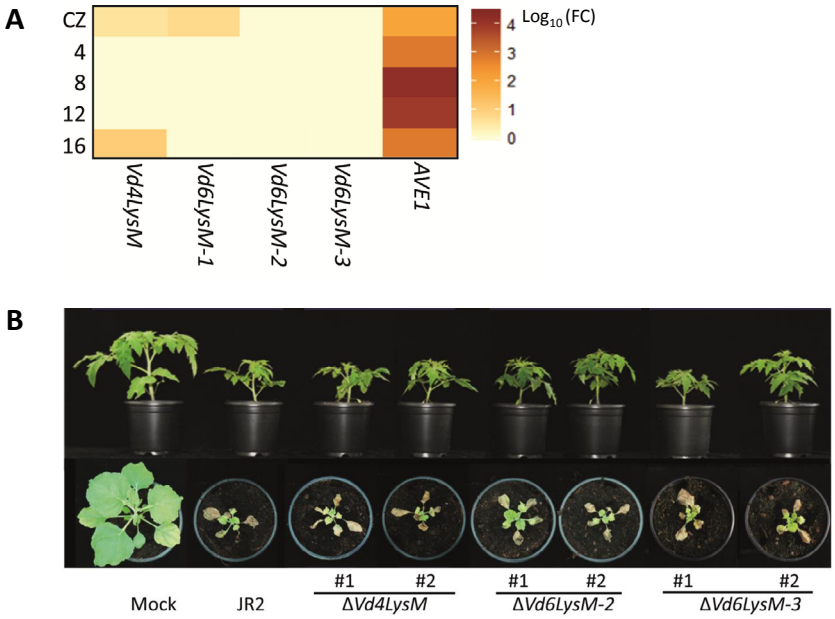

Figure 3. Core *VdLysM* effector genes do not contribute to virulence of *Verticillium dahliae* strain JR2 on tomato.

A: *VdLysM* effector gene expression and expression of the *Ave1* effector gene during growth of strain JR2 in Czapek Dox minimal medium (CZ) and during colonization of *N. benthamiana* at 4, 8, 12 and 16 days post inoculation. The expression profile is based on RNA sequencing data (de Jonge et al., 2012; Faino et al., 2013). FC is fold change.

B: Infection assay of wild-type and *VdLysM* effector gene deletion strains on tomato and *N. benthamiana* plants showing two deletion strains for each *VdLysM* effector gene. At 21 days post inoculation pictures of one representative plant were taken, out of six that were inoculated with the same *V. dahliae* genotype. The infection assays on tomato and *N. benthamiana* were repeated three times with similar results.

strain JR2 was evaluated upon targeted deletion. Multiple deletion strains were obtained for LysM effector genes *Vd4LysM*, *Vd6LysM-2* and *Vd6LysM-3*, while attempts to obtain a deletion mutant for *Vd6LysM-1* repeatedly failed. Importantly, none of the deletion strains that were obtained showed any phenotypic deviations from the wild-type JR2 strain in vitro (data not shown). Subsequent virulence assays revealed that tomato plants inoculated with the wild-type strain and those inoculated with two independent deletion strains for *Vd4LysM*, *Vd6LysM-2* and *Vd6LysM-3* showed similar disease development. The timing and degree of stunting of the plants and wilting of the leaves was similar upon inoculation with the various genotypes (Fig. 3B). Similarly, also upon inoculation of *N. benthamiana* no differences in disease development could be observed between the *VdLysM* deletion strains and the wild-type (Fig. 3B). Thus, the lack of *VdLysM* effector gene expression and the observation that *VdLysM* deletion strains are not affected in virulence on tomato and *N. benthamiana*, strongly suggests that core the VdLysM effectors do not play an important role during host colonization.

Lineage-specific Vd2LysM effector is required for full virulence on tomato

It has previously been demonstrated that LS effectors of the *V. dahliae* strain JR2 contribute significantly to virulence on tomato (de Jonge et al., 2013). This finding suggests that LS regions of individual *V. dahliae* genotypes are important for the development of aggressiveness on particular host plants. Intriguingly one LysM effector gene, *VDAG_05180*, was originally identified in strain VdLs17 but could not be found in strain JR2 nor in any of the other nine additional *V. dahliae* strains that were sequenced, revealing that *VDAG_05180* is an LS effector gene. Therefore, it was tested whether the LS effector encoded by *VDAG_05180*, designated Vd2LysM (with two LysM domains), contributes to virulence of strain VdLs17. The expression of *Vd2LysM* during infection of *V. dahliae* strain VdLs17 on tomato and on *N. benthamiana* was investigated using real-time PCR, and expression was detected at each of the time points monitored, with peaks at six and eight days after inoculation, respectively (Fig. 4A). To test whether Vd2LysM contributes to virulence, *Vd2LysM* deletion strains were generated, none of which showed morphological aberrancies *in vitro* when compared with the VdLs17 wild-type strain (Fig. 4B). In contrast to the core LysM effector genes, which did not appear to contribute to fungal virulence upon targeted deletion in the JR2 strain, the *Vd2LysM* deletion strains showed significantly reduced virulence upon inoculation on tomato when compared with the wild-type strain VdLs17 (Fig. 4C). The plants that were inoculated with the wild-type strain showed stronger stunting than the plants that were inoculated with two independent *ΔVd2LysM* strains (Fig. 4C). In accordance with the reduced symptom development, real-time PCR quantification of fungal biomass showed that the *ΔVd2LysM* strains produced significantly less biomass than the wild-type (Fig. 4D). These results show that Vd2LysM plays a role in virulence of *V. dahliae*

on tomato, and confirms that LS effectors are not only important determinants of virulence in *V. dahliae* strain JR2 (de Jonge et al., 2013), but also in strain VdLs17.

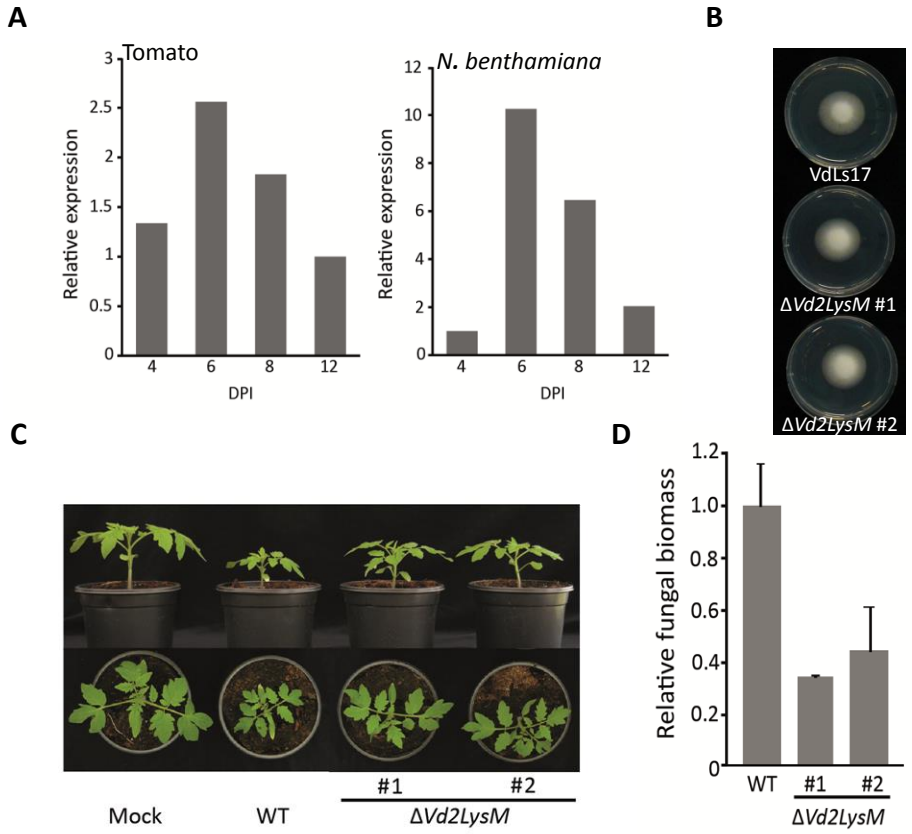

Figure 4. Lineage-specific LysM effector Vd2LysM contributes to virulence of *Verticillium dahliae* strain VdLs17 on tomato.

A: *Vd2LysM* expression during colonization of *V. dahliae* strain VdLs17 on tomato and *N. benthamiana* plants at 4,6,8 and 12 days post inoculation (DPI).

B: Morphology of wild-type *V. dahliae* strain VdLs17 and two *Vd2LysM* deletion strains after 7 days of incubation on PDA medium at room temperature.

C: Pictures of representative tomato plants out of eight plants that were either mock-inoculated or inoculated with wild-type *V. dahliae* strain VdLs17 and two deletion strains of *Vd2LysM* at 12 days post inoculation. The infection assay was repeated tree times with similar results.

D: Fungal biomass accumulation in tomato plants inoculated with wild-type strain VdLs17 and *Vd2LysM* deletion strains at 12 days post inoculation. Error bars represent the standard error of three replicate experiments.

In planta-produced Vd2LysM binds chitin

Previously characterized LysM effectors of fungal plant pathogens were demonstrated to bind chitin (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012). Therefore, the chitin-binding ability of Vd2LysM was tested. To this end, Vd2LysM was heterologously produced in the yeast *Pichia pastoris*, which has previously been used for production of LysM effectors from other fungi (Kombrink, 2012). Subsequently, the purified Vd2LysM protein was used in affinity precipitation assays with the insoluble carbohydrates chitin, chitosan, xylan and cellulose. We observed that Vd2LysM precipitated with all carbohydrates tested (Fig. 5A), which could suggest that the protein precipitates by itself rather than bind to any of these carbohydrates. To investigate this further, Vd2LysM was subjected to glycan-array analysis to test binding affinity for ~600 glycans, taking *C. fulvum* Ecp6 as a control. As demonstrated previously, Ecp6 specifically bound to the chitin oligosaccharides $(GlcNAc)_3$, $(GlcNAc)_5$ and $(GlcNAc)_6$ that are present on the array (de Jonge et al., 2010). In contrast, Vd2LysM did not bind to any of the glycans on the array (Supplemental Fig. 1). Thus, *P. pastoris*-produced Vd2LysM likely precipitated spontaneously in the affinity precipitation assay.

Recently, a crystal structure was obtained of *C. fulvum* Ecp6 that was heterologously produced in *P. pastoris*. Remarkably, the structure of the protein was found to contain

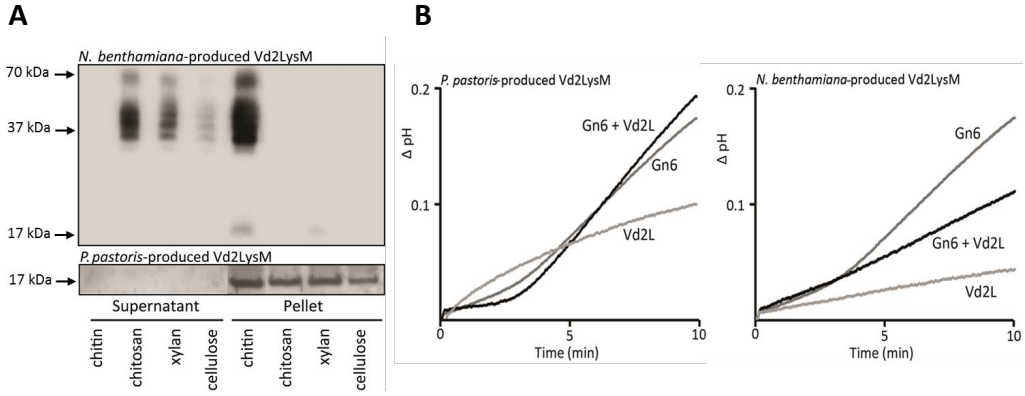

Figure 5. Vd2LysM is a chitin-binding protein that supresses chitin-induced immune responses.

A: Vd2LysM produced in *Pichia pastoris* and *in planta* was used in affinity precipitation experiments with insoluble polysaccharides chitin, chitosan, xylan or cellulose. After SDS-PAGE and Coomassie staining for *Pichia pastoris*-produced Vd2LysM or western blot analysis for *in planta*-produced Vd2LysM, the proteins are observed in the insoluble pellet fraction or in the supernatant fraction. Vd2LysM produced in *Pichia pastoris* is observed in the insoluble pellet fraction of all polysaccharides. Vd2LysM produced *in planta* is precipitated with chitin but not with chitosan, xylan or cellulose.

B: pH-shift measurements in a tomato cell suspension after addition of 1 nM chitin or 1 nM chitin that is pre-incubated with 10 nM Vd2LysM produced in either *Pichia pastoris* or *in planta*. The graphs represent a representative experiment out of two to four experiments with similar results.

chitin, likely derived from *P. pastoris* during protein production, in a binding groove that was composed of two LysM domains (Sánchez-Vallet et al., 2013). To exclude the possibility that potential Vd2LysM substrate binding sites were occupied by *P. pastoris* chitin, this protein was produced in *N. benthamiana* as plants are devoid of chitin. The purified protein was subsequently tested for chitin-binding ability in an affinity precipitation assay using chitin, chitosan, xylan and cellulose. This time, Vd2LysM bound to chitin, but not to any of the other carbohydrates (Fig. 5A). Furthermore, we observed that *N. benthamiana*-produced Vd2LysM migrates as multiple bands on gel, which were not visible with *P. pastoris*-produced Vd2LysM that migrates as a single band of the expected 17 kDa. This may indicate that Vd2LysM produced in *N. benthamiana* forms oligomers.

Vd2LysM suppresses chitin-induced immune responses

Previously, LysM effectors from various fungal plant pathogens were demonstrated to suppress the chitin-induced pH-shift in a tomato cell suspension, which is indicative for the ability of the effector to perturb chitin-induced host immune responses (Felix et al., 1993; de Jonge et al., 2010, Marshall et al., 2011, Mentlak et al., 2012). Both *in planta*-produced and *P. pastoris*-produced Vd2LysM were tested for this capacity as described previously (de Jonge et al., 2010). Interestingly, the *P. pastoris*-produced Vd2LysM that is not able to bind chitin was not able to suppress the chitin-induced pH-shift, whereas Vd2LysM produced in *N. benthamiana* that is able to bind chitin suppressed the chitin-induced immune response (Fig. 5B). These results suggest that Vd2LysM plays a role in suppression of chitin-triggered immune responses during *V. dahliae* colonization of tomato.

As the *M. graminicola* LysM effectors Mg3LysM and Mg1LysM were previously demonstrated to protect fungal hyphae against degradation by plant hydrolytic enzymes (Marshall et al., 2011), we also intended to test Vd2LysM for this activity. However, the yield of *in planta*-produced Vd2LysM was too low to obtain the required protein concentration. Therefore, we cannot conclude whether Vd2LysM has the ability to protect hyphae of *V. dahliae* during host colonization against hydrolysis by host enzymes.

Prediction of the three-dimensional structure of Vd2LysM suggests that its two LysM domains do not cooperatively bind chitin

Structural analysis of Ecp6 revealed that the first and third LysM domain cooperatively bind one chitin molecule with ultra-high (pM) affinity through intramolecular LysM dimerization, which allows Ecp6 to compete with plant receptors for chitin binding (Sánchez-Vallet et al., 2013). An alignment of the individual LysM domains of Ecp6 and Vd2LysM shows high overall sequence similarity, with a lower degree of similarity in the C-terminal

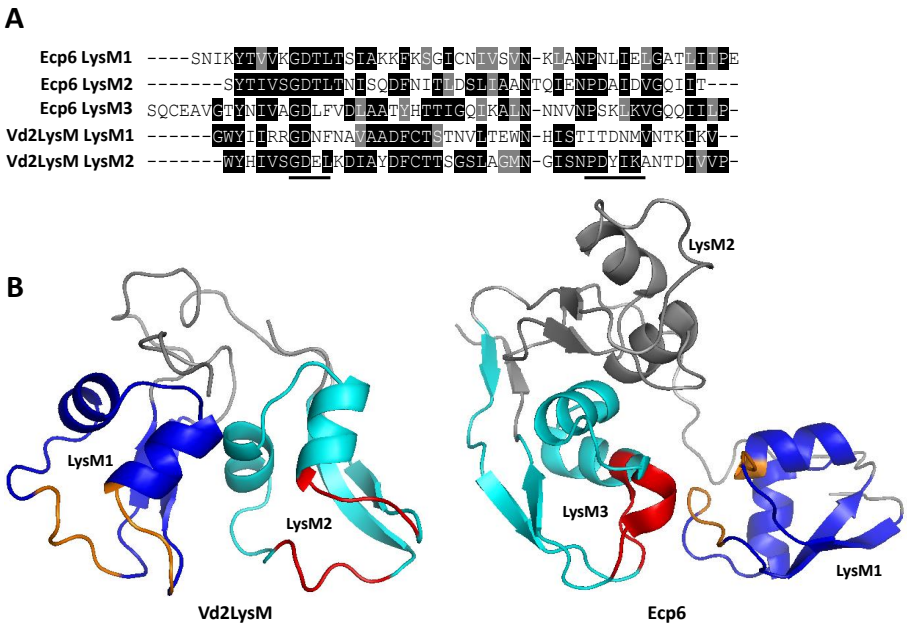

Figure 6. Predicted protein structure of Vd2LysM.

A: ClustalW alignment of the LysM domains of Ecp6 and Vd2LysM. Underlined are the amino acids that are implicated in chitin binding.

B: The predicted three-dimensional structure of Vd2LysM as generated with I-TASSER, showing the global arrangement of the two LysM domains of Vd2LysM. The regions that together form one chitin-binding site in LysM1 and one in LysM2 are indicated in orange and red, respectively. The previously determined structure of Ecp6 shows the chitin-binding loops of LysM1 and LysM3 indicated in orange and red, respectively, shaping a binding groove that binds one chitin molecule (Sánchez-Vallet et al., 2013).

part of the first LysM domain of Vd2LysM (Fig. 6A). To assess whether intramolecular LysM dimerization could also occur between the LysM domains of Vd2LysM, its overall structure was predicted using I-TASSER (Zhang, 2008; Roy et al., 2010; Roy et al., 2012), resulting in a structure model with a confidence (C) score of 0.34. Typically, the C-score is between -5 and 2 and a model with a C-score higher than -1.5 generally has a correct fold (Roy et al., 2010). The structure model indicates that the two LysM domains are oriented such that the two chitin-binding sites of the LysM domains are facing outward of the effector molecule and cannot cooperate to bind a single chitin molecule (Fig. 6B). Likely, the linker between these domains is too short to facilitate intramolecular dimerization of the LysM domains. Thus, Vd2LysM likely cannot bind chitin in an ultra-high affinity binding groove between two LysM domains of the same molecule, as observed in Ecp6. Therefore, the mechanism by which Vd2LysM perturbs the activation of chitin-triggered immunity needs further investigation.

LysM domains of Vd2LysM are more similar to LysM domains of previously characterized LysM effectors than to LysM domains of VdLysM effectors

Vd2LysM is the only LysM effector of *V. dahliae* for which a role in virulence could be shown, and that is able to suppress chitin-induced immunity in a similar fashion as previously described for Ecp6, Mg3LysM, Slp1 and ChELP1 (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012; Chapter 2). Therefore, the similarity of Vd2LysM to the previously characterized plant pathogen LysM effectors and the core VdLysM effectors was investigated. To this end, the individual LysM domains of these effectors were used to generate a similarity matrix using a ClustalW multiple sequence alignment of these do-

Table 1. The LysM domains of Vd2LysM are divergent from the LysM domains of the core VdLysM effectors.

LysM * effector	LysM domain	Vd2LysM LysM 1	Vd2LysM LysM2
Vd2LysM	1		
Vd2LysM	2	0.63	
Ecp6	1	0.56	0.59
Ecp6	3	0.53	0.61
Mg3L	1	0.51	0.63
ChELP1	1	0.51	0.61
ChELP2	2	0.53	0.56
Slp1	2	0.51	0.56
Slp1	1	0.51	0.56
ChELP2	1	0.51	0.54
Mg3L	3	0.51	0.53
Ecp6	2	0.44	0.56
Mg3L	2	0.46	0.47
Vd6LysM-1	1	0.46	0.46
ChELP1	2	0.44	0.47
Vd6LysM-1	2	0.44	0.47
Mg1L	1	0.42	0.47
Vd6LysM-1	3	0.44	0.44
Vd6LysM-1	5	0.42	0.42
Vd6LysM-1	6	0.41	0.37
Vd6LysM-2	5	0.44	0.34
Vd4LysM	3	0.39	0.37
Vd4LysM	4	0.41	0.36
Vd6LysM-2	6	0.42	0.32
Vd6LysM-2	3	0.36	0.39
Vd6LysM-1	4	0.36	0.39
Vd4LysM	2	0.37	0.37
Vd6LysM-3	6	0.39	0.34
Vd6LysM-2	1	0.34	0.39
Vd6LysM-3	3	0.37	0.32
Vd6LysM-3	4	0.37	0.32
Vd6LysM-3	5	0.37	0.32
Vd6LysM-2	2	0.36	0.34
Vd6LysM-3	2	0.34	0.32
Vd6LysM-3	1	0.32	0.34
Vd6LysM-2	4	0.34	0.31
Vd4LysM	1	0.32	0.27

degree of identity#
0.63
0.27

***** LysM domain sequences are derived from Vd2LysM, from the core VdLysM effectors and from previously characterized LysM effectors *Cladosporium fulvum* Ecp6, *Mycosphaerella graminicola* Mg1LysM and Mg3LysM, *Magnaporthe oryzae* Slp1, *Colletotrichum higginsianum* ChELP1 and ChELP2.
The identity is based on a ClustalW alignment of the individual LysM domain sequences (0.63=63% identity).

mains. Remarkably, the LysM domains of Vd2LysM are more similar to LysM domains of Ecp6, Mg3LysM, Slp1 and ChELP1 than to the LysM domains of the core VdLysM effectors (Table 1). This might be an indication that Vd2LysM is derived from another ancestor than the core VdLysM effector genes.

Discussion

In this chapter we describe the characterization of LysM effectors of *Verticillium dahliae*. Previously, comparative genomics has been performed with eleven *V. dahliae* isolates and it appeared that four VdLysM effector genes occur in the core genome of all these strains (de Jonge et al., 2012, 2013). In addition, a LS VdLysM effector gene is present in strain VdLs17 that does not occur in any of the other sequenced strains. We initially reasoned that the ubiquitous occurrence of the four core LysM effector genes suggests that these genes are required for host colonization by *V. dahliae*. However, according to the results described in this chapter a role in virulence cannot be attributed to these core LysM effectors, whereas we demonstrated that LS effector Vd2LysM contributes to virulence of strain VdLs17. Interestingly, several effector genes that are located in LS regions of strain JR2, including Ave1, were previously demonstrated to contribute to virulence of strain JR2 on tomato (de Jonge et al., 2013). The observation that specifically Vd2LysM contributes to virulence of strain VdLs17 confirms that LS effector genes of *V. dahliae* are important for fungal aggressiveness.

LysM effectors are found in fungal species with various lifestyles, but have only been studied as virulence factors of plant pathogenic fungi thus far (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012; Chapter 2). Moreover, they were only studied in foliar pathogens with a (relatively) narrow host range: *C. fulvum* (Ecp6), *M. graminicola* (Mg1LysM and Mg3LysM), *M. oryzae* (Slp1) and *C. higginsianum* (ChELP1 and ChELP2) (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012, Chapter 2). In contrast, *V. dahliae* is a soil-borne vascular plant pathogen that infects a broad range of host plants. The expression of the previously characterized LysM effectors is induced during the early stages of infection of the respective pathogens, when evasion of recognition by the host is in particular important to facilitate tissue colonization. The expression of *Vd2LysM* peaks at six days after inoculation which is when *V. dahliae* biomass is accumulating in the xylem and before wilting and necrosis of host tissue is visible (Fig. 4A) (Fradin and Thomma, 2006). Ecp6, Mg3LysM, Slp1 and ChELP1 were demonstrated to function as suppressors of chitin-induced host immunity and, similar to these proteins, Vd2LysM suppresses chitin-induced medium alkalinisation in a tomato cell suspension. This strongly suggests that Vd2LysM contributes to virulence of *V. dahliae* through perturbing the activation of chitin-triggered host immunity.

Considering the widespread occurrence of chitin receptor homologues in plant spe-

cies, and the observation that LysM effectors of diverse plant pathogens have the ability to suppress chitin-triggered immunity, it seems that this ability is fundamental for fungal plant pathogens to establish infection on their hosts. As all, except one, of the sequenced *V. dahliae* strains are pathogenic on tomato, these strains likely employ other molecules than Vd2LysM to interfere with chitin-triggered immunity. These molecules may act as functional homologs of Vd2LysM that perturb the activation of chitin-triggered immunity through their chitin-binding activity. In addition, *V. dahliae* may employ other mechanisms to prevent the activation of chitin-triggered immune responses, as has been observed in other fungal species. For example, the α-1,3-glucan synthase gene was demonstrated to be important for virulence of several fungal pathogens, as the α-1,3-glucan layer around hyphae reduces the accessibility of fungal chitin to host chitinases, and consequently prevents release of free chitin fragments (Fujikawa et al., 2012). In addition, fungal species were found to secrete chitin deacetylases that convert chitin into chitosan, which is a poor inducer of immune responses (Gough and Cullimore, 2011). Such strategies to prevent chitin perception by plant hosts might be important for *V. dahliae* pathogenicity as well, in addition to which strain VdLs17 employs Vd2LysM as an extra tool to perturb the activation of chitin-triggered immune responses.

Although the chitin receptor in tomato has not been identified, it is anticipated that tomato perceives chitin through homologues of chitin receptors that have been characterized and that contain extracellular LysM domains (Kombrink et al., 2011). As according to the structure prediction of Vd2LysM intramolecular dimerization of its two LysM domains is not likely, two hypotheses might explain how Vd2LysM perturbs the activation of immune signalling (Fig. 6). Firstly, intermolecular dimerization of LysM domains from two Vd2LysM molecules, rather than intramolecular dimerization, might result in a high affinity binding site that allows Vd2LysM to compete with host receptors through sequestration of chitin fragments. This hypothesis is supported by the indication that chitin is already bound to *P. pastoris*-produced Vd2LysM, which was similarly observed for the ultra-high affinity binding groove between LysM1 and LysM3 in Ecp6, but not for the binding site that is formed by LysM2 in the same molecule. Alternatively, the two LysM domains of Vd2LysM individually might bind chitin with lower affinity, as demonstrated for the second LysM domain of Ecp6. As it has been suggested that chitin-induced immune receptor dimerization is required for the activation of immune signalling, chitin binding by LysM effectors may also perturb chitin-induced immunity through interference with this dimerization (Liu et al., 2012; Sánchez-Vallet et al., 2013).

In conclusion, it is demonstrated that a lineage specific LysM effector of *V. dahliae* contributes to virulence, whereas four LysM effectors that are present in the core genome seem not to play a role during host colonization. However, it is tempting to speculate that LysM effectors play another role in fungal life. Therefore, it might be worthwhile to investigate whether the core LysM effectors contribute to fungal growth in other stages of the

V. dahliae life cycle that were not covered in this study, for example during saprophytic growth or during survival as resting structure in the soil. As *Vd4LysM* and *Vd6LysM-3* encode LysM effectors with either a reduced or increased number of LysM domains in various strains of *V. dahliae* (Fig. 2B), it should be taken into account that this variation might affect LysM effector functioning. Moreover, it is possible that only one of the Vd4LysM or Vd6LysM-3 variants is functional and therefore it would be interesting to also investigate these LysM effectors in other strains of *V. dahliae* than in JR2. However, until a biological role has been found for (a variant of) these LysM effectors, it is difficult to determine why loss or gain of LysM domains has occurred in these LysM effector proteins.

Materials and methods

Functional analysis of *VdLysM* effector genes

VdLysM effector gene deletion strains were generated by amplifying flanking sequences of the coding sequences using the primer sets presented in Supplemental Table 2. PCR products were subsequently cloned into pRF-HU2 (Frandsen et al. 2008). *V. dahliae* transformation and subsequent inoculations on tomato (cv. Motelle and MoneyMaker) plants to assess the virulence of the knock-out mutants were performed as described (Fradin et al. 2009). In one experiment six to eight plants were used per inoculation with wild-type or deletion strains and the experiment was repeated at least three times. Plants were regularly inspected and representative plants were photographed at 12 and 21 days post-inoculation (DPI). For biomass quantification, the roots and stem below the cotyledons of four plants per *V. dahliae* genotype were flash-frozen in liquid nitrogen. The samples were ground to powder, of which an aliquot was used for DNA isolation (Fulton et al. 1995). Real-time PCR was conducted with primer sets SlRub-F1/SlRub-F2 for tomato *RuBisCo* and VdGAPDH-F/VdGAPDH-R for *V. dahliae GAPDH* (Supplemental Table 1). For expression analyses, 3-week-old *Nicotiana benthamiana* plants were inoculated with strain VdLs17 as previously described (Fradin et al. 2009), harvested at 4, 6, 8 and 12 DPI, and flash-frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Kit (Qiagen) and cDNA was synthesized by SuperScript III (Invitrogen). Real-time PCR was conducted with primer sets Q-VdGAPDH-F/Q-VdGAPDH-R for *V. dahliae GAPDH* and Q-Vd2LysM-F/Q-Vd2LysM-R for *V. dahliae Vd2LysM* (Supplemental Table 2).

Similarity analyses of LysM domains

ClustalW was used to align amino acid sequences of the LysM domains of individual effector homologs. Subsequently, the alignments were used in the SIAS website (http://imed.med.ucm.es/Tools/sias.html) to generate identity matrices of LysM domain sequences in which all domains are compared to all domains in the alignment, resulting in sequence identity percentages between them.

Heterologous expression in *Pichia pastoris*

Vd2LysM was cloned into *P. pastoris* expression vector pPic9 (Invitrogen) after performing PCR using primers to add the N-terminal HIS- and FLAG-tag and *Eco*RI and *Not*I restriction sites for directional cloning (Supplementary Table 2). Subsequently, *P. pastoris* strain GS115 was transformed and a selected clone was cultured in a fermentor (Bioflo 3000) as described previously (Rooney et al., 2005). After removal of cells and concentration of the culture medium the HIS-tagged protein was purified using a Ni-NTA column (Qiagen) according to the manufacturer's protocol. The final protein concentration was determined spectrophotometrically at 280 nm and confirmed using the Pierce BCA Protein Assay Kit (Thermo Scientific, U.S.A.) with bovine serum albumin (BSA) as a standard.

***In planta* production of Vd2LysM**

PCR was performed to add *Nhe*I and *Sac*I restriction sites to *Vd2LysM*. Using directional cloning, *Vd2LysM* was cloned into vector pHYG, a modified version of the expression vector pMDC32 (Curtis and Grossniklaus, 2003). The expression vector was subsequently transferred into *Agrobacterium tumefaciens* strain MOG101. Infiltration of four- to five-week-old *Nicotiana benthamiana* with *Agrobacterium tumefaciens* was performed as described (Westerhof et al., 2012). Four days after infiltration, leaves were harvested and flash-frozen in liquid nitrogen. Plant material was ground and homogenized in ice-cold extraction buffer (1% v/v Tween-20, 2% w/v immobilized polyvinylpolypyrrolidone (PVPP), 300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, 1 mM Dithiothreitol (DTT), pH = 7,4) using 2 ml/g fresh plant material. After 30 min of homogenizing at 4°C, the crude extract was centrifuged at 16.000 x g at 4°C. The supernatant was further cleaned using a miracloth filter, and a Ni-NTA Superflow column (Qiagen) was used to purify the HIS tagged protein, according to the manufacturer's protocol. The final protein concentration was determined spectrophotometrically at 280 nm and confirmed using the Pierce BCA Protein Assay Kit (Thermo Scientific, U.S.A.) with bovine serum albumin (BSA) as a standard.

Affinity precipitation assays

These assays were performed as described (van den Burg et al., 2006) with 100 µg/ml Vd2LysM. The protein was incubated at RT for 1 hr with 3 mg of insoluble polysaccharides while gently rocking.

Structure prediction of Vd2LysM

A model for Vd2LysM was created using the I-TASSER website for protein structure and function predictions (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Zhang, 2008; Roy et al., 2010; Roy et al., 2012) using the Vd2LysM amino acid sequence without the sequence encoding the predicted signal peptide.

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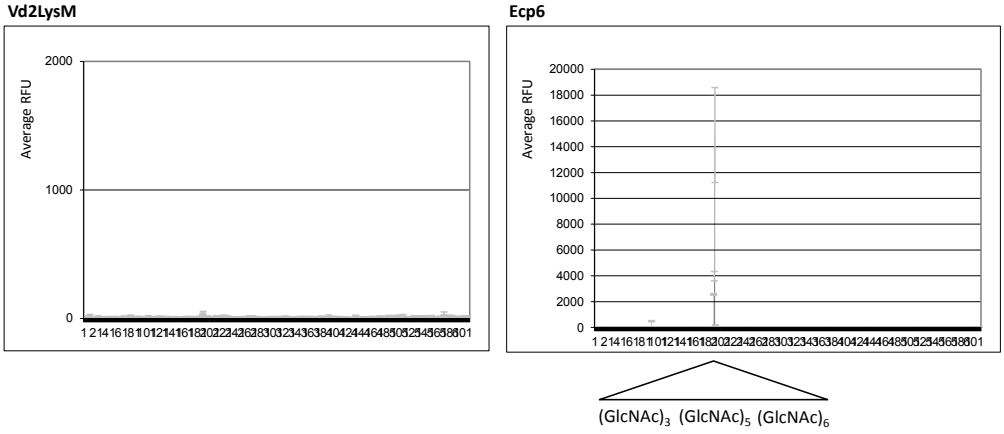
Supplemental data

Supplemental Table 1. ***Verticillium dahliae*** **strains used in this study**

Strain	Race	Originating host	Location	Year of collection	Tomato[#]	Tobacco*	Arabidopsis*
JR2	1	Tomato	ON, Canada	<1995	++	√	√
CBS381.66	1	Tomato	QC, Canada	1963	++	√	√
St14.01	1	Pistachio	CA, USA	?	++	√	√
St.100	n.a.	Soil	Belgium	?	-	√	√
DVD-3	2	Potato	Canada	1993	+	√	√
DVD-31	2	Tomato	Canada	1993	+	√	√
DVD-161	2	Potato	ON, Canada	1993	+/-	√	√
DVD-S26	2	Soil	Canada	1994	+	√	√
DVD-S29	2	Soil	Canada	1994	+/-	-	√
DVD-S94	2	Soil	Canada	1996	+/-	√	√
VdLs17	2	Lettuce	USA, CA	1995 ~ 2001	+/-	√	√

[#]Aggressiveness on susceptible tomato ranging from highly aggressive (++; severe stunting, wilting and chlorosis/necrosis), to aggressive (+; stunting and intermediate wilting), mildly aggressive (+/-; intermediate stunting, mild wilting) and non-pathogenic (-; no symptoms visible).
*Ability to infect this host (√) or not (-)



Supplemental Fig. 1. Histogram of *P. pastoris*-produced Vd2LysM and Ecp6 showing the quantified protein binding to the glycan array with on the y-axes the measured relative fluorescence units (RFU).

Supplemental Table 2. Primers used in this study

Primer name	Primer sequence 5'-3'	For
Vd4LysM-RB_F	GGACTTAAUGTAGCTTTGGAGAGATGAGG	KO Vd4LysM
Vd4LysM-RB_R	GGGTTTAAUCGTAGATCTTCTGGAGAACC	KO Vd4LysM
Vd4LysM-LB_F	GGTCTTAAUTAGAGGTTGAAGGAGAGGAG	KO Vd4LysM
Vd4LysM-LB_R	GGCATTAAUTGTACTGGCAATGACAATCT	KO Vd4LysM
Vd6LysM-1-RB_F	GGACTTAAUAGACGGCTGTATAGGTTGAT	KO Vd6LysM-1
Vd6LysM-1-RB_R	GGGTTTAAUACTACGTGCCTGCCTACTAC	KO Vd6LysM-1
Vd6LysM-1-LB_F	GGTCTTAAUCGGCGATTAATCTCTTAACT	KO Vd6LysM-1
Vd6LysM-1-LB_R	GGCATTAAUACGAATCTGACAAGAAAAGG	KO Vd6LysM-1
Vd6LysM-2-RB_F	GGACTTAAUGGAGTTGGAAAGGTCAGTAG	KO Vd6LysM-2
Vd6LysM-2-RB_R	GGGTTTAAUTCAATCAACAAGAGACAGGA	KO Vd6LysM-2
Vd6LysM-2-LB_F	GGTCTTAAUAGTTCATGAAGACGGAGAAG	KO Vd6LysM-2
Vd6LysM-2-LB_R	GGCATTAAUCATTCATGTCTAGCGAGGTA	KO Vd6LysM-2
Vd6LysM3-RB_F	GGACTTAAUAGGAGCTGTAAGGACTGAAG	KO Vd6LysM3
Vd6LysM3-RB_R	GGGTTTAAUTCAAGATAAACCACGAGAGA	KO Vd6LysM3
Vd6LysM3-LB_F	GGTCTTAAUCATGACACGACAGATAGGAG	KO Vd6LysM3
Vd6LysM3-LB_R	GGCATTAAUTTTACATAATGGCGAGAGTG	KO Vd6LysM3
Vd2LysM-RB_F	GGACTTAAUAGTTTTGCCTGACAGTAGGT	KO Vd2LysM
Vd2LysM-RB_R	GGGTTTAAUAATTGATAGTGAACGGCTTC	KO Vd2LysM
Vd2LysM-LB_F	GGTCTTAAUGGTTTTCTTACGCCAGTATC	KO Vd2LysM
Vd2LysM-LB_R	GGCATTAAUTTGTCTGACATGTTTCTCGT	KO Vd2LysM
Q-Vd2LysM-F	CCGAAGGACATGCAGTCATACCGG	Expression Vd2LysM
Q-Vd2LysM-R	TGCTGATATGGTTCCATTCCGTGAGG	Expression Vd2LysM
Q-VdGAPDH-F	CGAGTCCACTGGTGTCTTCA	Vd2LysM expression+ biomass VdLs17
Q-VdGAPDH-R	CCCTCAACGATGGTGAACTT	Vd2LysM expression+ biomass VdLs17
Q-Slrub-F	GAACAGTTTCTCACTGTTGAC	Biomass VdLs17
Q-Slrub-R	CGTGAGAACCATAAGTCACC	Biomass VdLs17
Vd2LysM-*Pich*-F	CGGTATGAATTCCATCATCATCATCATCCCGACTACAAGG ACGACGATGACAAGTACCGAAGGACATGCAGTCATAC	Vd2LysM *Pichia* production
Vd2LysM-*Pich*-R	CGTCTAGCGGCCGCTTAGTTCCAGCTGCACGGC	Vd2LysM *Pichia* production
Vd2LysM-plant-F	ACTAGTTACCGAAGGACATGCAGTCATAC	Vd2LysM *in planta* production
Vd2LysM-plant-R	GAGCTCTTAGTTCCAGCTGCACGGC	Vd2LysM *in planta* production

Chapter 5

LysM effectors play a role in fungal interactions with bacteria

Abstract

Previously, LysM effectors were demonstrated to contribute to virulence of various fungal plant pathogens through their ability to interfere with host immune responses. However, the presence of LysM effector genes in the genomes of fungi with a saprophytic lifestyle suggests that LysM effectors contribute to fungal physiology in other manners as well. Potentially, LysM effectors play a role in the interaction of fungi with other microbes in the environment, which could even be relevant for plant pathogenic fungi that encounter other microbes at the site of host infection. This hypothesis is investigated using an *in vitro* system in which the fungal species *Trichoderma viride* is co-cultivated with the bacterial species *Bacillus subtilis* and *Lysobacter capsici*, which aggregate with fungal hyphae and cause reduction of fungal growth, respectively. Incubation of germinating *T. viride* conidiospores with LysM effectors prior to the addition of bacteria indicate that LysM effectors play a role in the interaction of fungi with bacteria.

Introduction

Fungi occur in diverse ecological niches where they secrete a variety of molecules that facilitate their establishment. This includes the secretion of effector proteins, which are most extensively studied in the interaction between fungal pathogens and their hosts where they play an essential role in the establishment of the infection (de Jonge et al., 2011). While host immune responses are activated upon pathogen invasion, effector proteins often function through interfering with such responses and, consequently, facilitate colonization (Dodds and Rathjen, 2010; de Jonge et al., 2011). Although fungal effectors are considered to be mostly species- or even lineage-specific, a class of effectors that occurs in many pathogenic fungi are LysM effectors (de Jonge and Thomma, 2009). LysM effectors are defined as secreted proteins that contain no recognizable domains apart from a varying number of lysin motifs (LysMs) (de Jonge and Thomma, 2009).

The fungal LysM effectors that are characterized thus far are secreted by various fungal plant pathogens that infect various hosts and were all demonstrated to bind chitin, an *N*-acetyl-D-glucosamine (GlcNAc) homopolymer (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012; Chapters 2,4). Chitin is a major component of the fungal cell wall and plays an important role in the interaction between fungal pathogens and their plant hosts. Plants do not produce chitin, but are able to perceive fungal chitin fragments by cell surface-localized receptor proteins that trigger the activation of immune responses to stop pathogen growth (Kaku et al., 2006; Kombrink et al., 2011). The LysM effector Ecp6 that is produced by the tomato pathogen *Cladosporium fulvum* was the first LysM effector that was demonstrated to contribute to pathogen virulence through perturbing the activation of chitin-triggered host immune responses (de Jonge et al., 2010). Subsequently, LysM

effectors of other fungal species were demonstrated to similarly suppress chitin-triggered immunity, including the LysM effector Mg3LysM secreted by the wheat pathogen *Mycosphaerella graminicola* (Marshall et al., 2011; Mentlak et al., 2010). In addition, Mg3LysM was demonstrated to protect fungal hyphae against degradation by hydrolytic enzymes that are produced by plants to stop fungal growth (Marshall et al., 2011). *M. graminicola* also secretes the LysM effector Mg1LysM which similarly protects fungal hyphae against plant hydrolytic enzymes, but which does not have the ability to suppress chitin-triggered immunity (Marshall et al., 2011).

Interestingly, LysM effector genes are not only found in the genomes of fungal pathogens, but also in fungi with a saprophytic lifestyle, which suggests that LysM effectors contribute to fungal growth in other conditions than during interactions with a host. Therefore, we hypothesized that LysM effectors might play a role during interactions with other microbes. In any niche, fungi encounter a community of microbes, including antagonistic species that inhibit fungal growth. Antagonism against fungi might be based on the release of toxins, antibiotics, volatiles and the secretion of hydrolytic enzymes that cause degradation of the fungal cell wall (de Boer et al., 2005). Antagonistic activity of bacteria and fungi is studied particularly in relation to biocontrol of fungal pathogens, focusing on antifungal activity of antagonists that decrease pathogen performance (Duffy et al., 2003). However, little is known about the molecular basis of microbial interactions. Moreover, although it is likely that fungi employ strategies to protect themselves from microbial antagonism, this is hardly investigated thus far (Duffy et al., 2003; Mathioni et al., 2013).

Thus far, the role of LysM effectors of *C. fulvum* and *M. graminicola* in the interaction with defense responses of plant hosts was evaluated. To investigate whether these LysM effectors potentially also play a role in the interaction with other microbes that are present in their niches, an *in vitro* system to assess the effect of these effectors outside a plant host was pursued. However, this leads to two complications, namely the fact that *C. fulvum* and *M. graminicola* (LysM) effectors are strictly induced *in planta* and not *in vitro* (Bolton et al., 2008; Marshall et al., 2011), and the fact that cell wall chitin is not exposed in many fungi grown *in vitro*, including *C. fulvum* (van de Burg et al., 2006; van Esse et al., 2007). To overcome these limitations, we pursued an experimental system with a fungus that has exposed cell wall chitin, and to which we heterologously produced LysM effector proteins of *C. fulvum* and *M. graminicola* can be added. As it was previously demonstrated that *Trichoderma viride* is sensitive to chitinase activity *in vitro* (van de Burg et al., 2006; van Esse et al., 2007), it was anticipated that this fungus may be a suitable test organism.

Results

Assessment of potential antagonistic effects of bacterial various species on *T. viride*

In order to develop an *in vitro* assay system that allows testing potential effects of LysM effectors on the interaction between fungi and bacteria, *T. viride* was exposed to bacteria *in vitro* and inspected for signs of interaction. To this end, a range of bacterial species was used including the mammalian gut commensal *Escherichia coli*, the plant pathogen *Pseudomonas syringae*, the soil inhabitant *Bacillus amiloliquefaciens*, the soil inhabitant and mammalian gut commensal *Bacillus subtilis* and the ubiquitous soil and water inhabitant *Lysobacter capsici* (Hirano and Upper, 1990; Park et al., 2008; Chen et al., 2009; Qin et al., 2010; Postma et al., 2010). The latter three species have previously been reported to act as biocontrol agents (Chen et al., 2009; Tesfagiorgis and Korsten, 2006; Liu et al., 2009).

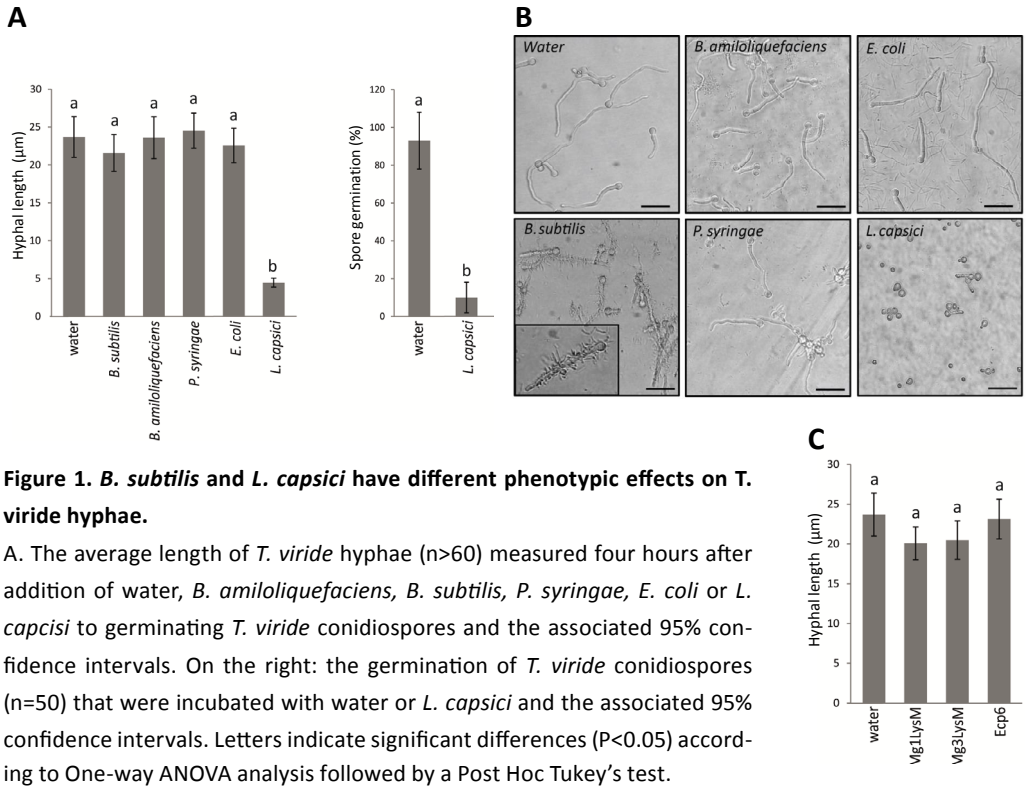


Figure 1. *B. subtilis* and *L. capsici* have different phenotypic effects on T. viride hyphae.

A. The average length of *T. viride* hyphae (n>60) measured four hours after addition of water, *B. amiloliquefaciens, B. subtilis, P. syringae, E. coli* or *L. capcisi* to germinating *T. viride* conidiospores and the associated 95% confidence intervals. On the right: the germination of *T. viride* conidiospores (n=50) that were incubated with water or *L. capsici* and the associated 95% confidence intervals. Letters indicate significant differences (P<0.05) according to One-way ANOVA analysis followed by a Post Hoc Tukey's test.

B. Representative pictures were taken six hours after addition of *B. amiloliquefaciens, B. subtilis, P. syringae, E. coli* or *L. capcisi* to germinating *T. viride* conidiospores. Scale bars represent 30 µm.

C. The average length of *T. viride* hyphae (n>60) measured four hours after addition of water or 20 µM of Mg1LysM, Mg3LysM or Ecp6 to germinating *T. viride* conidiospores and the associated 95% confidence intervals. No significant difference was determined with One-way ANOVA analysis.

T. viride conidiospores were incubated overnight in a microtiter plate containing PDB. Upon germination of the first conidiospores after approximately sixteen hours, bacteria were added at a final OD_{600} of 0.02 and the growing hyphae were monitored over time. Co-cultivation of *T. viride* with the bacterial species *E. coli*, *P. syringae* and *B. amiloliquefaciens* did not lead to any visible effect on the fungal hyphae, and the length of the hyphae that were exposed to the bacteria did not show significant differences when compared with the length of hyphae that were not exposed to bacteria (Fig. 1A,B). Strikingly, we observed that *B. subtilis* aggregated with the fungal hyphae, which became visible within one hour after addition of the bacteria, and which resulted in complete coverage of the hyphae with bacteria. Nevertheless, aggregation of *B. subtilis* with the fungal hyphae did not result in reduced growth of these hyphae (Fig. 1A). In contrast, the presence of *L. capsici* in the growth medium significantly impaired spore germination and caused severe growth reduction of the fungal hyphae (Fig. 1A,B). Thus, visual phenotypes were observed upon co-cultivation of *T. viride* with the bacterial species *B. subtilis* and *L. capsici in vitro*. Therefore, these bacterial species were used in follow-up experiments to investigate whether LysM effectors have the potential to play a role in fungal interactions with bacteria.

Ecp6, Mg3LysM and Mg1LysM perturb attachment of *B. subtilis* cells to *T. viride* hyphae

To investigate whether the addition of LysM effectors affects the growth of *T. viride*, the LysM effectors Ecp6, Mg1LysM or Mg3LysM were added to germinating *T. viride* conidiospores at a final concentration of 20 µM. As expected, no visual effect was observed after four hours of incubation, which demonstrates that these LysM effectors do not affect hyphal growth of *T. viride* (Fig. 1C). To investigate whether LysM effectors affect the aggregation of *B. subtilis* with *T. viride* hyphae, germinating conidiospores were incubated with Mg1LysM, Mg3LysM or Ecp6 for one hr prior to the addition of the bacteria. Interestingly, after pre-incubation of *T. viride* with either of these LysM effectors, hardly any aggregation of bacterial cells with the fungal hyphae was observed. Whereas initially 20 µM of the LysM effectors was used, even a concentration of 1 µM of the LysM effectors was sufficient to prevent bacterial aggregation (Fig. 2A). As a control, the hyphae were incubated with 1 µM of BSA, which did not affect association of *B. subtilis* with hyphae (Fig. 2A).

Chitin-binding ability has previously been described for several bacterial species, including binding to chitin of fungal hyphae (Brandl et al., 2012). As we expect that chitin is exposed in the cell wall of *T. viride* (van den Burg et al., 2006), it was tested whether *B. subtilis* has the ability to bind chitin. To this end, *B. subtilis* was incubated with chitin beads (New England Biolabs, Beverly, U.S.A.). To visualize the bacteria, Syto BC nucleic acid stain (Invitrogen, Carlsbad, U.S.A) was added prior to microscopical evaluation of the chitin beads. Clear localization of the bacteria on the bead surface was observed after one hour of incubation of the beads with the bacteria (Fig. 2B). Also in this assay,

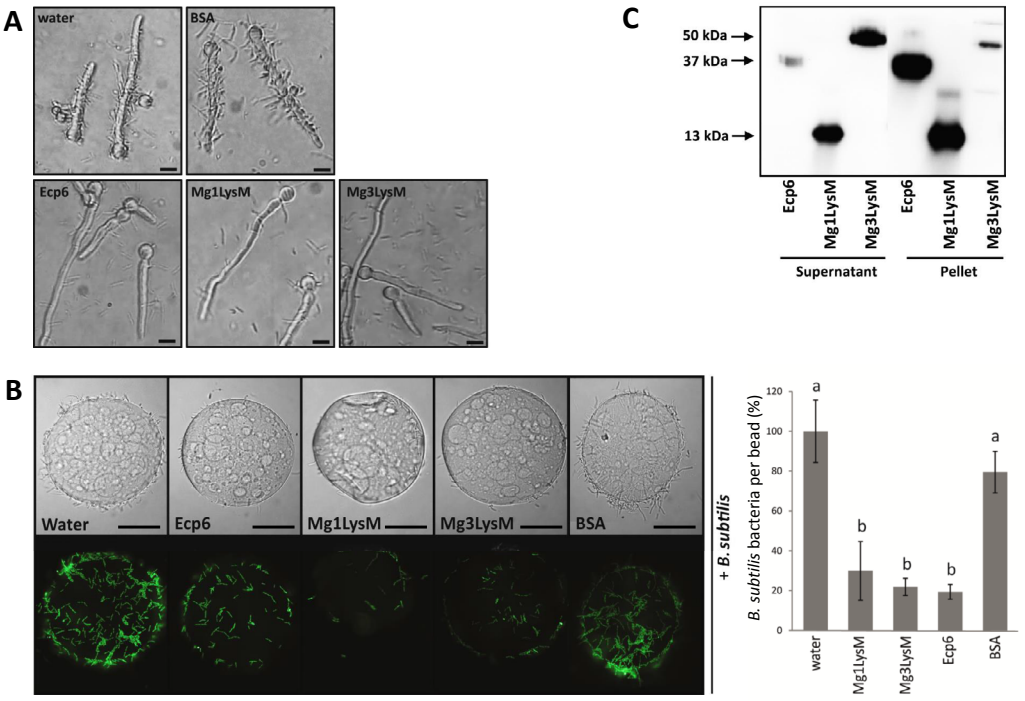


Figure 2. LysM effectors perturb aggregation of *B. subtilis* with *T. viride* hyphae.

A. Germinating *T. viride* conidiospores were incubated with water or 1 µM of BSA, Mg1LysM, Mg3LysM or Ecp6 prior to addition of *B. subtilis*. Representative pictures were made after four to six hours of incubation. The experiment was repeated at least three times with similar results. Scale bars represent 10 µm.

B. Chitin beads were incubated with water or 5 µM of BSA, Mg1LysM, Mg3LysM or Ecp6 prior to addition of *B. subtilis*. After approximately two hours of incubation, the localization of *B. subtilis* was observed microscopically. Representative pictures out of eight pictures of examined beads are shown. On the right: the percentage of bacteria on the beads (n>5) that were incubated with Mg1LysM, Mg3LysM, Ecp6 or BSA relative to the number of bacteria on the beads that were incubated with water. The associated 95% confidence intervals are indicated. Letters indicate significant differences ($P<0.05$) according to One-way ANOVA analysis followed by a Post Hoc Tukey's test. The experiment was performed twice with similar results.

C. Affinity precipitation of Mg1LysM, Mg3LysM or Ecp6 with *B. subtilis*. Effector proteins remaining in the supernatant (S) and the insoluble chitin pellet (P) after SDS-PAGE and western blot analysis is shown.

incubation of chitin beads with Ecp6, Mg3LysM or Mg1LysM prior to addition of the bacteria greatly reduced the aggregation of bacteria on the beads, whereas incubation with BSA did not affect the aggregation (Fig. 2B). Together, these results indicate that *B. subtilis* can localize to chitin in the *T. viride* cell wall.

Chitin-binding has previously been demonstrated for Mg1LysM, Mg3LysM and Ecp6 (de Jonge et al., 2010; Marshall et al., 2011), and a valid hypothesis could be that these LysM effectors bind to fungal cell wall chitin and thus block access of the bacteria to the chitin. Nevertheless, it cannot be excluded that the LysM effectors bind to the bacterial cells, rather than to the fungal hyphae, in order to prevent bacterial aggregation at the fungal hyphae. To test whether the LysM effectors directly bind *B. subtilis* cell walls, a pull down experiment was performed in which bacterial cells were pelleted in the presence of Mg1LysM, Mg3LysM or Ecp6. Interestingly, all three LysM effectors were observed in the pellet fraction, which indicates that the LysM effectors bound to *B. subtilis*.

Mg1LysM impairs growth reduction of *T. viride* hyphae by *L. capsici*

Whereas localization of *B. subtilis* to *T. viride* did not significantly affect hyphal growth, conidiospore germination and length of *T. viride* hyphae was severely reduced upon incubation with *L. capsici* (Fig. 1A). As the density of *L. capsici* of OD_{600} of 0.02 was detrimental to most of hyphae, it was anticipated that a potential role for LysM effectors might not be visible and, therefore, a final OD_{600} of 0.004 of *L. capsici* was used to investigate whether Mg1LysM, Mg3LysM or Ecp6 play a role in protection of hyphae against growth inhibition by *L. capsici*. Incubation of *T. viride* conidiospores with Mg1LysM prior to the addition of *L. capsici* resulted in a higher germination percentage when compared with conidiospores that were incubated with water, Mg3LysM or Ecp6 (Fig. 3B). Evaluation of the hyphal length of germinating *T. viride* conidia at two and four hours after addition of *L. capsici* showed that pre-incubation with Mg1LysM reduced the inhibitory effect of *L. capsici*, whereas pre-incubation with Ecp6 and Mg3LysM did not significantly affect the growth inhibition that is caused by *L. capsici* (Fig. 3A).

As only Mg1LysM was demonstrated to decrease the effect of *L. capsici* on hyphal growth and conidiospore germination, we investigated whether Mg1LysM has the ability to bind to *L. capsici*. To this end, Ecp6, Mg3LysM and Mg1LysM were used in a pull down assay with *L. capsici*. Only Mg1LysM was observed to precipitate with bacterial cells, whereas Ecp6 and Mg3LysM were only present in the supernatant fraction (Fig. 4A). Previously, glycan-array analysis of Ecp6 revealed that Ecp6 only binds chitin oligomers $GlcNAc_3$, $GlcNAc_4$ and $GlcNAc_6$ (de Jonge et al., 2010). To further investigate the potential differential ligand specificity of the three LysM effectors used in this study, also Mg1LysM and Mg3LysM were subjected to glycan-array analysis to test their binding specificity for ~600 glycans (Blixt et al., 2004). As expected, both Mg1LysM and Mg3LysM bound to the chitin oligomers that are present on the array: $GlcNAc_3$, $GlcNAc_4$ and $GlcNAc_6$. In contrast to Ecp6 (de Jonge et al., 2010) and Mg3LysM, Mg1LysM was found to also bind Neu5Ac-$GlcNAc_3$ (Fig. 4B). Neu5Ac is N-acetylneuraminicacid which is the most common form of sialic acid that is found at the terminal of animal and bacterial cell-surface glycan molecules. Together,

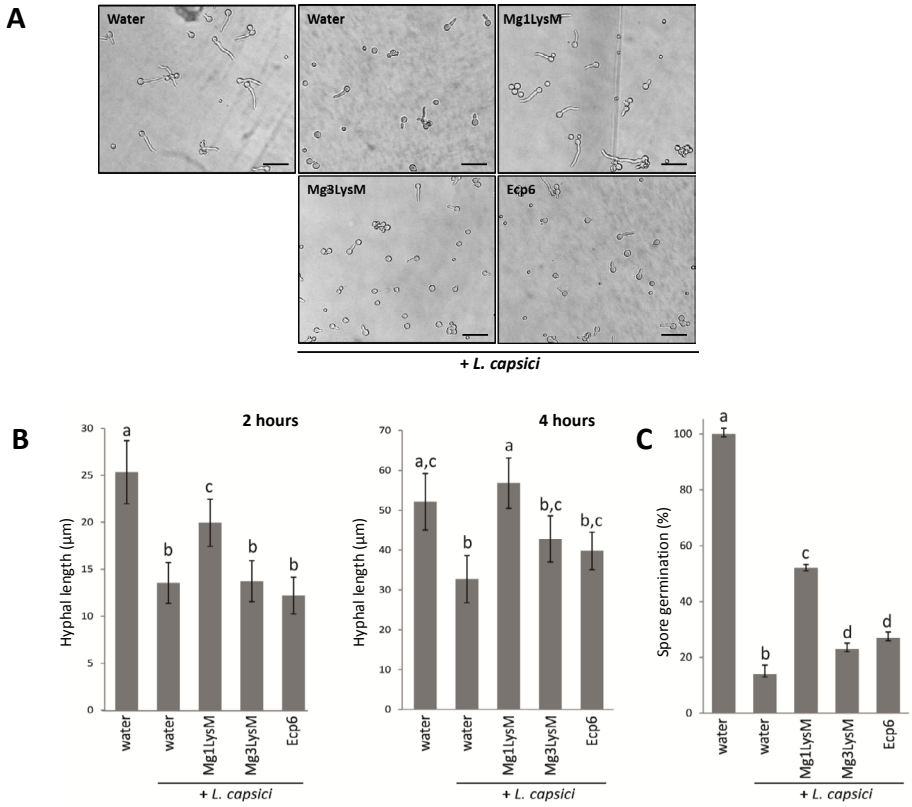


Figure 3. Mg1LysM decreases the growth reduction of *T. viride* hyphae caused by *L. capsici*.

A. Germinating *T. viride* conidiospores were incubated with water or 20 µM of Mg1LysM, Mg3LysM or Ecp6 prior to addition of *L. capsici*. Representative pictures of three technical replicates were made after two hours of incubation. The experiment was repeated twice with similar results. Scale bars represent 20 µm.

B. Length of *T. viride* hyphae (n>60) relative to the *T. viride* spore size at two and four hours after addition of *L. capsici* and the associated 95% confidence intervals. Prior to addition of *L. capsici*, germinating *T. viride* conidiospores were incubated with water or 20 µM of Mg1LysM, Mg3LysM or Ecp6. At least 20 hyphae were measured per well in three different wells for each treatment. Letters indicate significant differences (P<0.05) according to One-way ANOVA analysis followed by a Post Hoc Tukey's test.

C. *T. viride* conidiospores were incubated with water or 20 µM of Ecp6, Mg1LysM or Mg3LysM prior to addition of *L. capsici*. The ratio of the germinated conidiospores over the total number of *T. viride* conidiospores was calculated after approximately five hours of incubation in a 96-well microtiter plate. At least 40 conidiospores per well were counted in three wells for each treatment and the associated 95% confidence intervals are indicated. Letters indicate significant differences (P<0.05) according to One-way ANOVA analysis followed by a Post Hoc Tukey's test.

these observations suggest that Mg1LysM decreases the antagonistic effect of *L. capsici* through direct interaction with the bacterial cells.

Discussion

In this chapter, a potential role for LysM effectors in the interaction of fungi with bacteria was investigated. In any environment, fungi will encounter other microbes that may compete for the same nutrient sources, or even display direct antagonistic activity. Thus, defense strategies against antagonistic microbes are likely to be important for fungal species with diverse lifestyles in order to establish themselves in their niches. Here, we explored a potential role for the LysM effector Ecp6 that is produced by *C. fulvum*, and Mg1LysM and Mg3LysM of *M. graminicola*, in the interaction of fungi with bacteria. However, as these LysM effectors are strictly induced *in planta* and not *in vitro* (Bolton et al., 2008; Marshall et al., 2011), an *in vitro* system that exploits the fungal species *T. viride* was pursued. Interestingly, it was observed that application of Mg1LysM to *T. viride* reduces the inhibitory effect of *L. capsici* on the growth of *T. viride* hyphae. This result shows that Mg1LysM has the potential to affect antagonistic bacteria that may occur during host colonization by *M. graminicola*. However, although it is tempting to speculate that Mg1LysM plays such role *in planta*, additional experiments are required to demonstrate this. *M. graminicola* penetrates the leafs of wheat plants quickly after spore germination, and encounters en-

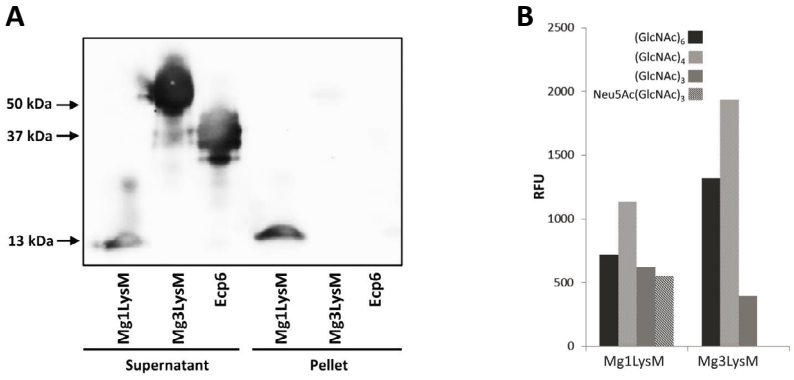


Figure 4. Mg1LysM binds to *L. capsici* cells and to sialic acid on a glycan array.

A. Affinity precipitation of Mg1LysM, Mg3LysM or Ecp6 with *L. capsici*. Effector proteins remaining in supernatant (S) and the insoluble chitin pellet (P) after SDS-PAGE and western blot analysis is shown.

B. Glycan array analysis showing the quantified protein binding to the glycan array with on the y-axes the measured relative fluorescence units (RFU). Mg1LysM and Mg3LysM bind to $GlcNAc_3$, $GlcNAc_4$ and $GlcNAc_6$. In addition, only Mg1LysM binds to Neu5Ac-$GlcNAc_3$.

dophytic bacteria during growth inside the apoplast, some of which may display antagonistic activity (Berg et al., 2005; Lodewijckx et al., 2013). Although it is demonstrated that endophytic bacteria can suppress fungal growth (Berg et al., 2005; Coombs et al., 2004), most experiments to test antagonism were performed *in vitro* and little is known about the actual interactions between endophytic bacteria and fungi *in planta* (Lodewijckx et al., 2013). To investigate a potential role for Mg1LysM in the interaction with antagonistic bacteria during host colonization by *M. graminicola*, infection assays using wild-type and *Mg1LysM* deletion strains (Marshall et al., 2011) should be performed on wheat plants and carefully inspected with respect to the capability of the fungal strains to establish their niche. Previously, the *Mg1LysM* deletion strain was shown to be as virulent as wild-type *M. graminicola* on wheat plants in inoculation experiments under controlled conditions (Marshall et al., 2011). However, this analysis was largely based on symptom analysis and fungal biomass accumulation was not monitored, making that subtle changes in pathogen accumulation may easily have been overlooked (Marshall et al., 2011). Furthermore, the contribution of Mg1LysM to fungal growth may depend on experimental conditions, and it is conceivable that the pathogen encounters other populations of endophytic bacteria in natural infections of wheat plants in the field. To further investigate a potential role for Mg1LysM, wild-type and *Mg1LysM* deletion strains can be inoculated onto wheat plants after infiltration of the leaves with *L. capsici*, and subsequently the virulence of the *Mg1LysM* deletion strain can be compared to the virulence of wild-type *M. graminicola* under these conditions. Nevertheless, as *L. capsici* has been isolated from the rhizosphere of pepper (Park et al., 2008), this bacterium will likely not be encountered in natural infections of wheat leaves. Thus, endophytes that naturally occur in wheat leaves should eventually be identified tested for their effect on growth of wild-type and *Mg1LysM* deletion strains *in planta*.

Initially, we aimed to set up an *in vitro* system to explore a potential role for LysM effectors in the interaction of fungi with bacterial antagonists. *L. capsici* was included as a previously described antagonist of various fungal species (Park et al., 2008; Postma et al., 2010). However, other antagonistic bacterial species might be used to investigate a role for LysM effectors in interactions between fungi and bacteria, including bacteria occurring in the niche of the fungal species that produce these LysM effectors. Although we focused on bacterial species, fungi likely encounter other antagonists, including other fungal species and nematodes. Investigations into a role of LysM effectors in fungal defense against antagonists should therefore be extended to other organisms.

Although we tested the role of exogenously applied LysM effectors from *C. fulvum* and *M. graminicola* upon addition to *T. viride*, it has to be taken into account that *T. viride* itself may also produce LysM effectors. No *T. viride* genome has been sequenced to date, but the related species *T. atroviride*, *T. reesii* and *T. virens* were all demonstrated to contain multiple LysM effector genes in their genomes (de Jonge and Thomma, 2009). Potentially, LysM

effector genes of *T. viride* are even induced *in vitro*, as no studies have been performed yet on LysM effector gene induction in saprophytic species. Thus, it should be taken into account that the effects of exogenously applied LysM effectors may be dampened or masked by the secretion of endogenous LysM effectors of *T. viride*.

According to the results described in this chapter, Ecp6, Mg1LysM and Mg3LysM prevent the aggregation of *B. subtilis* with *T. viride* hyphae. However, the aggregation of bacteria with the hyphae did not lead to visible effects on hyphal physiology, and therefore it cannot be concluded whether the LysM effector-mediated prevention of bacterial aggregation to fungal hyphae is relevant for fungal niche establishment. The association of bacteria to fungal hyphae is not specific for *B. subtilis* and *T. viride,* but has been described for various bacterial and fungal species (de Boer et al., 2005). Therefore, it should be investigated whether association of other bacterial species to hyphae affects fungal growth. Subsequently, it can be tested whether prevention of aggregation to hyphae by LysM effectors has beneficial effects for the fungus. It is conceivable that particular bacterial species need to attach to fungal hyphae to feed on them or to display their antifungal activity (Leveau and Preston, 2007). Again, additional experiments should reveal whether LysM effectors play a role in fungal interactions with bacterial antagonists under natural conditions.

The observation that Ecp6, Mg1LysM and Mg3LysM prevent the aggregation of *B. subtilis* with *T. viride* hyphae whereas only Mg1LysM decreased hyphal growth reduction caused by *L. capsici,* might indicate that LysM effectors affect bacteria in different manners. Mg1LysM, Mg3LysM and Ecp6 bind to *B. subtilis* in a pull down assay and to chitin oligosaccharides on a glycan-array. Therefore, the ability of LysM effectors to interfere with association of *B. subtilis* to fungal hypae can depend on binding of LysM effectors to chitin in the fungal cell wall such that the chitin is not available for bacterial attachment. Alternatively, LysM effectors perturb association of *B. subtilis* with the fungal hyphae through their ability to bind *B. subtilis* cell walls. *B. subtilis* is a Gram-positive species which contains peptidoglycan (PGN) in the outer layer of the cell wall. PGN is a chitin-related glycan composed of alternating residues of *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine (GlcNAc) and is a ligand of various LysM proteins of bacteria and plants (Buist et al.; Willman et al., 2011). In contrast, only Mg1LysM was demonstrated to bind to the Gram negative bacterial species *L. capsici*. Furthermore, only this LysM effector bound to Neu5Ac-$GlcNAc_3$ on the glycan array. Neu5Ac (*N*-acetylneuraminic acid, or sialic acid) is the most common member of the family of sialic acids that occur especially on animal cells, but is also found on the cell wall of many bacterial species as decoration of lipopolysaccharides (Severi et al., 2007). However, it should be noted that the bacterial species that are found to contain sialic acid are mostly animal pathogenic bacteria and that little is known about the presence of sialic acid on lipopolysaccharides of non-animal pathogenic bacteria. Therefore, it can be hypothesized that sialic acid on the cell wall of *L. capsici* mediates binding of Mg1LysM, although it is currently unknown whether this bacterial species contains sialic

acid at all. In addition to a role in the interaction with bacteria, the ability to bind sialic acid might be relevant for LysM effectors of animal pathogens in the interaction with their hosts. Whereas plants are generally thought not to contain sialic acid (Séveno et al., 2004), sialic acid occurs as terminal monomer of glycans on many animal cells and plays an important role in physiological processes, including immune signalling. Therefore, it could be hypothesized that fungal animal pathogens secrete sialic acid-binding LysM effectors in order to interfere with immune signalling of their animal hosts.

In conclusion, the results in this chapter are a starting point for further investigations into a role for LysM effectors in the interaction of fungi with antagonistic bacteria, which could reveal that LysM effectors contribute to fungal growth through other manners than through interfering with host immune responses.

Acknowledgement

We want to thank Joeke Postma and Irene de Bruijn for providing the *L. capsici* isolate.

Materials and methods

Fungal and bacterial growth

T. viride conidiospores were harvested in potato dextrose broth (PDB) from 5- to 10-day-old potato dextrose agar (PDA) plates grown at room temperature (RT) and dilutions of 10^5 spores/ml were made. In 96-wells microtiter plates, 45 µl of the spore suspension was incubated overnight at RT. The next day, bacteria *B. amiloliquefaciens*, *B. subtilis*, *P. syringae*, *E. coli* or *L. capcisi* that were grown on LB plates at RT or at 37˚C (*B. subtilis*) were resuspended from plate in liquid LB and diluted to an OD_{600} of 1. Subsequently bacteria were added to the germinating *T. viride* conidiospores at a final OD_{600} of 0.02 and the wells were monitored for six hours. To test the effect of LysM effectors, *T. viride* conidiospores were similarly harvested and incubated overnight and germinating conidiospores were incubated for 1-2 hours with water, Ecp6, Mg1LysM or Mg3LysM prior to the addition of *B. subtilis* or *L. capsici.* An end concentration of 1 µM of the LysM effectors was used in the experiment with *B. subtilis* and 20 µM in the experiment with *L. capsici*. The hyphae were monitored up to six hours after addition of the bacteria. Each treatment of fungal conidiospores was done in triplicate per experiment and the experiments were repeated at least three times. Hyphae were measured with NIS-Elements AR 2. 3 software (Nikon Instruments Inc., Melville, USA.

Chitin bead assay

Chitin beads (New England Biolabs, Beverly, U.S.A.) were washed three times with Milli-Q water to re-

move the ethanol in which the beads were stored. Subsequently, aliquots of 45 µl of the beads were incubated in a 96-well microtiter plate with 5 µM of the proteins for 1 hr while shaking at 200 rpm. A *B. subtilis* suspension in LB with an OD_{600} of 1 was obtained by resuspending bacterial cells from an overnight PDA plate culture grown at 37˚C and 1 µl was added to the beads to obtain an end concentration with an OD_{600} of 0.02. After two hours of incubation the bacteria were stained with a green fluorescent nucleic acid stain Syto-BC (Invitrogen, Carlsbad, U.S.A) and visualised using a Nikon eclipse 90*i* UV microscope and NIS-Elements AR 2. 3 software (Nikon Instruments Inc., Melville, USA).

Pull down assay

The affinity precipitation assay with *L. capsici* was performed using bacteria that were grown on PDA plate as described above. Bacteria were suspended by pipetting tap water on the plate and the suspension was pelleted by centrifugation at 11.000 rpm. The pellet was washed once with water and the bacteria were resuspended in 1 ml of water or 1 ml of 20 µM of Ecp6, Mg1LysM or Mg3LysM in water. The HISFLAG-tagged proteins and *L capsici* cells were incubated at RT for 1 hr while gently rocking. The insoluble fraction was pelleted by centrifugation (5 min, 13,000 × g) and the supernatant was collected. The insoluble fraction was washed three times with water and subsequently boiled in 150 µl of 1% SDS. Presence of protein in supernatant and pellet was examined by SDS-PAGE followed by immuno-blotting.

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Chapter 6

General discussion

This chapter is adapted from:

Kombrink A., Thomma B.P.H.J. (2013) LysM Effectors: secreted proteins supporting fungal life. *PLoS Pathogens* **9** e1003769.

Introduction

Fungi occupy a plethora of niches and play essential roles in diverse environments through decomposition of organic material as saprophytes or through establishment of symbiotic relationships with plants and animals that range from mutually beneficial to pathogenic. During colonization of their niches, fungi secrete proteins that include carbohydrate-degrading enzymes to feed on complex molecules and effectors that mediate the establishment of interactions with host organisms (Lowe and Howlett, 2012). To overcome host immunity, pathogens secrete effector molecules that manipulate host physiology, including immune responses, to support host colonization (de Jonge et al., 2011 Thomma et al., 2011). Likely, also other microbes that establish intimate relationships with host plants, such as mutualistic symbiotic microbes and endophytes, secrete effectors to establish their association. Although effectors are typically thought to be species- or even lineage-specific, some effectors are widespread among pathogens, such as the necrosis- and ethylene-inducing-like proteins (NLPs) that are widely spread in bacteria, fungi, and oomycetes (de Jonge et al., 2011; Gijzen and Nürnberger, 2006). Several studies have shown that NLPs contribute to pathogen virulence through phytotoxic activity, but recent work has revealed that some NLPs act in processes other than pathogenicity, such as fungal growth and sporulation (Santhanam et al., 2013). A more recently identified class of conserved effectors are LysM effectors: fungal effectors that carry no recognizable protein domains other than lysin motifs (LysMs) (de Jonge and Thomma, 2009). Intriguingly, like NLPs, LysM effectors occur in both pathogenic and in non-pathogenic fungi. The LysM effectors that are functionally characterized thus far are secreted by various plant pathogenic fungi. *Cladosporium fulvum* (Ecp6), *Mycosphaerella graminicola* (Mg1LysM and Mg3LysM), *Magnaporthe oryzae* (Slp1) and *Colletotrichum higginsianum* (ChELP1 and ChELP2) are foliar pathogens of tomato, wheat, rice and brassicaceae plants respectively, whereas the soil-borne pathogen *Verticillium dahliae* (Vd2LysMs) is a vascular pathogen on a broad range of host plants (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012; Chapter 2; Chapter 4).

Plant pathogen LysM effectors: virulence factors through interactions with chitin

Fungal chitin in plant immunity

The fungal cell wall consists of a complex network of glycoproteins and polysaccharides, including glucans and chitin (Bowman and Free, 2006; Latgé, 2007). Chitin is a homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) and is essential for the integrity of the cell wall. During colonization of plant hosts, fungal cell wall-derived chitin functions as microbe-

associated molecular pattern MAMP that is recognized by host cell surface receptors and triggers an immune response. Several plasma membrane localized chitin receptors have been identified in plants that all contain extracellular LysM domains (Felix et al., 1993; Kaku et al., 2006; Kombrink et al., 2011). Furthermore, fungal cell wall chitin is a target of plant chitinases that act in fungal immunity. Exochitinases are not detrimental to fungal hyphae but release chitin oligosaccharide MAMPs from fungal cell walls that can induce host immune responses. This includes the secretion of endochitinases that are aimed to degrade the fungal cell wall and eventually causes lysis of the hyphal cells (Felix et al., 1993; Schlumbaum et al., 1986). Thus, fungal chitin plays an essential role in plant immunity against fungal pathogens. However, fungal pathogens employ strategies that interfere with chitin-triggered immunity, which includes the secretion of LysM effectors, to enable host colonization.

Suppression of chitin-induced immune responses

LysM effectors produced by several plant pathogenic fungi were demonstrated to suppress chitin-triggered immunity and to be important for virulence of the pathogens on their hosts. Therefore, deregulation of chitin-triggered immunity seems to be an important function of LysM effectors (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012). The first identified LysM effector Ecp6 is secreted by tomato leaf mold pathogen *Cladosporium fulvum* and it was proposed that Ecp6 functions by sequestration of cell wall-derived chitin fragments that would otherwise be perceived by host immune receptors (de Jonge et al., 2010) (Fig. 1). The crystal structure of Ecp6 showed that two LysM domains (LysM1 and LysM3) collectively bind a single chitin molecule (Sánchez-Vallet et al., 2013) (Fig. 1). This ligand-induced composite binding groove is deeply buried in the effector and displays ultra-high (picomolar) chitin binding affinity, which is significantly higher than that of plant immune receptors (Sánchez-Vallet et al., 2013). Through analysis of a crystal structure of the Arabidopsis chitin elicitor receptor kinase (AtCERK1) it was previously demonstrated that only one of the three LysM domains in this immune receptor binds chitin (Liu et al., 2012). Moreover, the structural orientation of the three LysM domains in AtCERK1 does not permit intramolecular LysM dimerization as observed in Ecp6 (Liu et al., 2012; Sánchez-Vallet et al., 2013). Interestingly, the singular LysM domain of Ecp6 that is not involved in the intramolecular composite binding site (LysM2) also contains a functional chitin-binding site (Fig. 1), and has the capacity to perturb chitin-induced immunity (de Jonge et al., 2010; Sánchez-Vallet et al., 2013). Since the chitin binding affinity of this singular LysM domain is significantly lower than that of the composite binding site, it is unlikely to deregulate chitin-induced immunity merely by chitin oligosaccharide sequestration. As it has been suggested that chitin-induced immune receptor dimerization is required for the activation of immune signalling, LysM2 may perturb chitin-induced immunity through

interference with this dimerization (Liu et al., 2012; Sánchez-Vallet et al., 2013) (Fig. 1, 2).

Interestingly, prediction of the three-dimensional structure of the *V. dahliae* LysM effector Vd2LysM, which has the ability to suppress chitin-induced immune responses, revealed that the two LysM domains of this LysM effector are oriented such that the two chitin-binding sites of the LysM domains are facing outward of the effector molecule and cannot cooperate to bind a single chitin molecule. In addition, the linker between these domains is too short to facilitate intramolecular dimerization of the LysM domains and therefore Vd2LysM likely cannot bind chitin in an ultra-high affinity binding groove between two LysM domains of the same molecule, as observed in Ecp6. (Chapter 4). Although it has not been investigated, other LysM effectors that suppress chitin-induced immunity and that contain two LysM domains, such as *C. higginsianum* ChELP1 and *M. oryzae* Slp1, might have a similar three dimensional structure as Vd2LysM (Chapter2; Mentlak et al., 2012). The two LysM domains of Vd2LysM, and possibly of ChELP1 and Slp1, might bind chitin individually with lower affinity than the composite binding site, similar to the second LysM domain of Ecp6. Alternatively, intermolecular dimerization of Vd2LysM creates a high affinity binding site that allows competition with host chitin receptors through sequestration of chitin oligosaccharides. Future experiments, including chitin-binding affinity measurements and determination of the protein structure through protein crystallisation might reveal the mechanism by which Vd2LysM, and possibly other LysM effectors with a similar global structure, perturb the activation of chitin-triggered immunity.

Protection of fungal cell wall chitin from hydrolytic plant enzymes

Interestingly, functional analysis of *M. graminicola* LysM effectors has revealed that they may have an additional function during host colonization (Marshall et al., 2011). *M. graminicola* Mg1LysM and Mg3LysM prevent hyphal lysis by plant chitinases whereas *C. fulvum* Ecp6, *M. oryzae* Slp1, *C. higginsianum* ChELPs and *V. dahliae* Vd2LysM do not have this capacity (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012; Chapter 2; Chapter 4) (Fig. 2). Thus, functional diversification of LysM effectors during host colonization has occurred in plant pathogens. The protection of fungal cell wall chitin against plant hydrolytic enzymes was previously demonstrated for *C. fulvum* Avr4, which does not contain LysM domains but binds chitin through an invertebrate chitin-binding domain (van den Burg et al., 2003; 2006). Furthermore, Avr4 was demonstrated to be important for virulence of *C. fulvum* (van Esse et al., 2006).

In addition to their protection against degradation by plant enzymes, *M. graminicola* LysM effectors and Avr4 might also inhibit the activation of chitin-triggered immunity through protection against the activity of non-detrimental exochitinases that release chitin MAMPs from the fungal cell wall. It is likely that MgLysMs and Avr4 decrease the accessibility of fungal chitin for these enzymes, resulting in a reduced release of chitin fragments

that could be perceived by host receptors. A complementary strategy is the synthesis of a α-1,3-glucan layer around hyphae, which was demonstrated to be important for virulence of several fungi, as it reduces the accessibility of fungal chitin to host chitinases, and consequently prevents the release of chitin fragments that may trigger an immune response (Fujikawa et al., 2012). The presence of α-1,3-glucan synthase genes is conserved among fungal plant pathogens, including M. oryzae and *M. graminicola*, which is a further indication that prevention of chitin-triggered host immune responses is essential for the ability of fungal plant pathogens to colonize their hosts.

LysM effectors as virulence factors of mammalian pathogens?

Genome mining revealed that LysM effectors are not confined to plant pathogens as, for instance, genomes of most (opportunistic) fungal pathogens of mammals contain LysM effector genes as well (de Jonge and Thomma, 2009). For instance, in the dermatophyte *Trichophyton rubrum*, causal agent of athlete's foot, as well as in related dermatophyte species, the gene family encoding LysM effectors appears to be expanded (Martinez et al., 2012). Similar to plants, mammals do not synthesize chitin but can respond to chitin with an immune response, which includes the production of chitinases (Lee et al., 2008). These observations tempt speculation that fungal pathogens of mammals secrete LysM effectors to deal with host immunity in a similar fashion as plant pathogens (Kombrink et al., 2011). Furthermore, allergies such as asthma are associated with fungal infections, although the underlying mechanisms presently remain unclear (Goldman and Vicencio, 2012). Emerging evidence suggests an important role for host chitinases that might mediate host responses to chitin and its derivatives (Goldman and Vicencio, 2012), which may again be influenced by fungal LysM effectors. However, the fact that chitin is not universally recognized as a MAMP in mammalian systems argues against the hypothesis that fungal mammalian

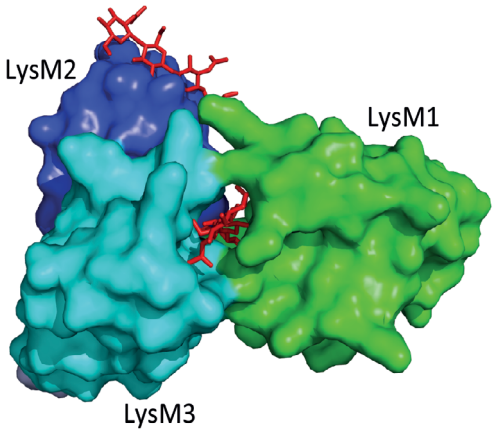

Figure 1. Three dimensional structure of the *Cladosporium fulvum* LysM effector Ecp6. Two LysM domains of Ecp6 (LysM1 and LysM3) cooperate to form a binding groove that binds a single chitin oligosaccharide molecule (chitin tetramer oligosaccharide in red) with picomolar affinity. The remaining, singular, LysM domain (LysM2) also has a functional chitin-binding site, although its affinity for chitin binding is significantly lower than that of the composite binding site.

pathogens secrete LysM effectors to establish infection (Mora-Montes et al., 2011). Furthermore, the genome of the human pathogenic yeast *Candida albicans*, as well as of most other *Candida* spp. that occur as opportunistic human pathogens, appears to lack LysM effector genes (de Jonge and Thomma, 2009). Similarly, in the genome of the skin-associated fungus *Malassezia globosa* that is responsible for the onset of dandruff and other skin disorders, and the fungus *Pneumocystis jirovecii* that causes pneumonia among immunocompromised hosts, no LysM effector genes are found (Xu et al., 2007; Cissé et al., 2012). Since many mammalian pathogens show a low degree of host adaptation and lack host specificity, it has been suggested that infection by mammalian fungal pathogens does not require effector activity (Lowe and Howlett, 2012). In contrast to plant pathogens, most fungal pathogens of mammals spend a considerable amount of their life cycle free-living in the environment and only infect mammalian hosts in an opportunistic manner. Thus, mammalian fungal pathogens may use their LysM effector homologues in processes other than host colonization, such as survival in the environment. The aforementioned absence of LysM effector genes in *Candida albicans*, *Malassezia globosa* and *Pneumocystis jirovecii*, which are among the few fungal species that are commensals of humans and animals and that do not occur free-living in the environment, seems to support this hypothesis (de Jonge and Thomma, 2009; Xu et al., 2007; Cissé et al., 2012).

LysM effectors of saprophytes: diverse possibilities

Considering that LysM effectors are ubiquitous in fungi, it could be argued that they might act in general physiological processes, such as cell wall modification. Fungi secrete lytic enzymes that break chitin polymers and in this manner maintain cell wall flexibility to allow hyphal growth, branching, morphogenesis, and spore germination. Recently, a *Trichoderma atroviride* LysM effector was found to be coexpressed with an adjacent chitinase gene (Seidl-Seiboth et al., 2013). Since addition of the purified LysM effector to *T. atroviride* inhibited spore germination *in vitro*, a role in hyphal growth was proposed for this LysM effector. However, further experimental evidence that includes targeted deletion of the LysM effector gene in *T. atroviride* is required to support such a role.

Their occurrence in saprophytes may furthermore suggest that LysM effectors contribute to growth in any fungal niche, as likely other microbes are encountered that compete for the same niche or may act as mycoparasites. In this respect, several hypotheses can be envisaged. Extrapolating the findings for LysM effectors of plant pathogens, LysM effectors may protect fungi against chitinases and other hydrolytic enzymes produced by mycoparasites. It was demonstrated that the localization of the bacterial species *Bacillus subtilis* to fungal hyphae is largely inhibited by LysM effectors, which might also be attributed to their chitin-binding ability (Chapter 5). Moreover, sequestration of cell wall-derived chitin oligosaccharides may be relevant if mycoparasites would be attracted by gradients of such

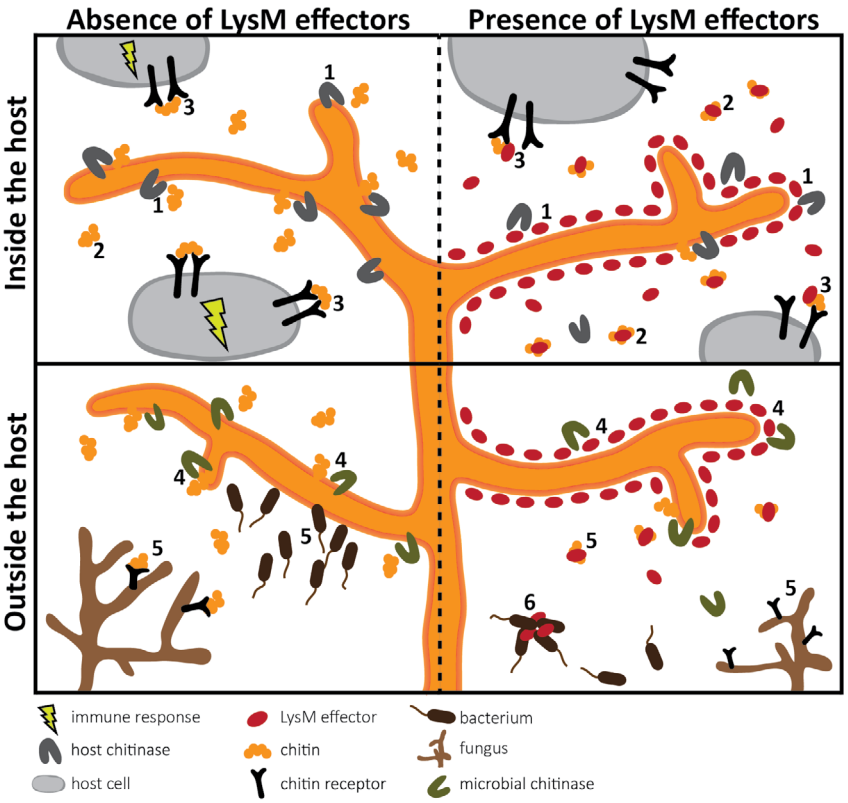

Figure 2. Overview of the diverse roles that fungal LysM effectors may play in fungal physiology. LysM effectors may act during host colonization (upper panels) and outside the host (lower panels). Pathogen LysM effectors have been implicated in two different pathogenicity-related processes (upper panels). Firstly, LysM effectors may protect fungal hyphae against degradation by hydrolytic enzymes secreted by the host (1). Secondly, LysM effectors may secure fungal cell wall-derived chitin fragments so that chitin cannot stimulate an immune response because LysM effectors efficiently scavenge chitin fragments (2), or interfere with host receptor activation by preventing ligand-induced dimerization (3). As LysM effectors also occur in non-pathogenic fungi (lower panels), they may protect fungal hyphae against hydrolytic enzymes secreted by mycoparasites (4). In addition, chitin sequestration might prevent attraction of such microbes (5). Some LysM effectors may recognize chitin-related carbohydrates such as peptidoglycan and immobilize bacterial competitors (6).

fragments (Fig. 2). One step further, LysM effectors may also have functions that are not associated with chitin binding. The observation that *M. graminicola* Mg1LysM, but not Mg3LysM or Ecp6, binds to sialic acid on a glycan array and to *Lysobacter capsici*, indicates that differences in substrate specificity occur among LysM effectors (Chapter 5). It appears that Mg1LysM has the ability to protect hyphae from antifungal activity displayed by *L. capsici*, although the mechanism by which Mg1LysM protects needs further investigation.

Originally, LysMs were identified in bacterial lysozymes (hence the name of the domain) that bind and hydrolyse peptidoglycan, a chitin-related glycan and a major component of bacterial cell walls (Buist et al., 2008). LysMs occur in various peptidoglycan-binding proteins, and thus it is conceivable that some LysM effectors bind peptidoglycan as well. Such LysM effectors may help fungi to affect bacterial competitors in their niches, for instance because they immobilize them in a similar fashion as antibodies do (Yang et al., 2006) (Fig. 2).

Concluding remarks

Fungal LysM effectors are versatile proteins that occur in fungal species with extremely divergent lifestyles. Conceivably, LysM effectors function in various ecological niches. In addition, even LysM effectors of plant pathogens that function in the same niche (the plant host) and that bind the same substrate (chitin) were demonstrated to have distinct roles in promoting fungal virulence (Kombrink et al., 2011; Marshall et al., 2011). Furthermore, pathogens interact with other microbes, both in the free-living stage and during colonization of their hosts where they may encounter opportunistic pathogens, commensals, and endophytes. In this respect it is interesting to note that the vascular wilt pathogen *Verticillium dahliae* contains four core LysM effectors that are present in eleven sequenced strains and one lineage specific (LS) effector that is only present in strain VdLs17 (de Jonge et al., 2013; Chapter 4). Interestingly, functional analysis revealed that only the LS LysM effector gene is induced *in planta* and contributes to pathogenicity, while the role of the core LysM effector genes still remains obscure (de Jonge et al., 2013; Chapter 4). *V. dahliae* is known to survive as a resting structure in the soil for decades in the absence of suitable host plants, and it is tempting to speculate that the core LysM effectors contribute to persistence of these structures through protection against microbial activity. Therefore, the study of LysM effectors of fungi that thrive in a variety of niches will reveal additional LysM effector functions that are relevant for pathogenic fungi as well.

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Summary

Chitin is a homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) that is abundantly present in nature and found as a major structural component in the fungal cell wall. In **Chapter 1**, the role of chitin as an important factor in the interaction between fungal pathogens and their plant hosts is discussed. As plants do not produce chitin, they evolved to recognize fungal chitin as a non-self molecule by plasma membrane receptors that can activate host immune responses to stop fungal growth. To overcome those host immune responses, fungal pathogens secrete effector molecules that manipulate host physiology, including immune responses, to support colonization. The chitin-binding Lysin motif (LysM) effector Ecp6 from the fungal tomato pathogen *Cladosporium fulvum* was previously demonstrated to contribute to virulence through interfering with the activation of chitin-induced host immune responses. Subsequently, LysM effector genes were found in the genomes of many fungal species.

In **Chapter 2** we describe the functional characterization of LysM effectors of the plant pathogenic fungi *Mycosphaerella graminicola*, *Magnaporthe oryzae* and *Colletotrichum higginsianum*, which cause leaf blotch disease of wheat, rice blast disease and anthracnose disease on Brassicaceae, respectively. This functional analysis revealed that the ability to perturb chitin-induced immunity is conserved among LysM effectors of these fungal plant pathogens. In addition, two LysM effectors that are secreted by *M. graminicola* were found to protect fungal hyphae against cell wall hydrolytic enzymes from plants, demonstrating that LysM effectors can contribute to virulence of fungal plant pathogens in multiple ways.

The *M. graminicola* LysM effector Mg3LysM and *C. fulvum* Ecp6 both contain three LysM domains and show a high overall similarity. However, whereas Mg3LysM can protect fungal hyphae against plant-derived cell wall hydrolytic enzymes, Ecp6 does not have this capacity. **Chapter 3** describes a functional analysis of the contribution of LysM domains of Mg3LysM to its protection ability. To this end a series of chimeric proteins were produced in which LysM domains of Mg3LysM were swapped with the corresponding LysM domain of Ecp6. Analysis of these chimeras indicated that protection against the hydrolytic activity of plant enzymes is mediated by the concerted activity of LysM1 and LysM3 in Mg3LysM.

LysM effectors do not only occur in foliar fungal plant pathogens, but also in soil-borne pathogens that infect their host through the roots. In **Chapter 4**, LysM effectors of the fungal soil-borne vascular wilt pathogen *Verticillium dahliae* are described. Comparative genomics of eleven *V. dahliae* strains revealed that four LysM effectors are found in the core genome, which are referred to as core VdLysM effectors. Intriguingly, for none of the core LysM effector genes expression could be monitored during host colonization, and targeted deletion could not reveal a role in virulence, suggesting that the core LysM effectors do not act as virulence factors during host colonization. In addition to the core genome, *V. dahliae* strains generally carry lineage-specific (LS) genomic regions. Interestingly, an

additional LysM effector gene (*Vd2LysM*) was found in an LS region of *V. dahliae* strain VdLs17 that is absent in all other sequenced *V. dahliae* strains. Remarkably, the LS effector Vd2LysM was found to contribute to virulence of strain VdLs17. Like the previously characterized plant pathogen LysM effectors, also Vd2LysM was found to bind chitin and suppress chitin-induced immune responses. These results indicate that Vd2LysM interferes with chitin-induced immunity during host colonization by *V. dahliae* strain VdLs17.

Thus far, LysM effectors were demonstrated to contribute to virulence of various fungal plant pathogens through their ability to interfere with host immune responses. However, the presence of LysM effector genes in the genomes of non-pathogenic fungi and fungi with a saprophytic lifestyle suggests that LysM effectors contribute to fungal physiology in other manners as well. In **Chapter 5** we investigated the hypothesis that LysM effectors play a role in the interaction of fungi with other microbes in the environment, which could even be relevant for plant pathogenic fungi that encounter other microbes at the site of host infection. To investigate this hypothesis, assays were developed that allow to assess the attachment and antagonistic effects of particular bacterial species on fungi by employing the fungus *Trichoderma viride*, as this species is known to have accessible cell wall chitin upon growth *in vitro*. Assays to assess bacterial attachment and antagonistic activity in the absence or presence of LysM effectors indicate that LysM effectors play a role in the protection of fungi against bacterial competitors.

In **Chapter 6**, the major results described in this thesis are discussed and a perspective on the (potential) roles of LysM effectors in fungi with different lifestyles, including pathogenic as well as non-pathogenic fungi, is presented.

Samenvatting

Chitine, een homopolymeer van *N*-acetyl-D-glucosamine (GlcNAc), komt veelvuldig voor in de natuur en vormt een belangrijke structurele component van celwanden van schimmels. In **Hoofdstuk 1** wordt de rol van chitine in de interactie tussen plantpathogene schimmels en hun gastheer besproken. Planten produceren zelf geen chitine, maar hebben receptoren op de celmembraan die chitine herkennen als "lichaamsvreemd" en het immuunsysteem activeren om dreigende infecties te voorkomen. Desondanks kunnen pathogene schimmels hun gastheer infecteren door effectormoleculen uit te scheiden die de fysiologie van deze gastheer manipuleren, inclusief het immuunsysteem. De schimmel die de bladvlekkenziekte van tomaat veroorzaakt, *Cladosporium fulvum*, produceert de effector Ecp6 die chitine-bindende Lysin motieven (LysMs) heeft en die bijdraagt aan virulentie door middel van het onderdrukken van chitine-geïnduceerde immuniteit in de gastheer. Genen die coderen voor homologen van Ecp6, ook wel LysM effectoren genaamd, komen in het DNA (het genoom) van veel schimmelsoorten voor.

Hoofdstuk 2 beschrijft de functionele karakterisering van LysM effectoren van de plantpathogene schimmels *Mycosphaerella graminicola*, *Magnaporthe oryzae* en *Colletotrichum higginsianum*, die respectievelijk bladvlekkenziekte van tarwe, rijstpest en kanker van kruisbloemigen veroorzaken. Er wordt aangetoond dat ook LysM effectoren van deze schimmels chitine binden en chitine-geïnduceerde immuniteit van hun gastheer kunnen onderdrukken. Daarnaast blijkt dat twee LysM effectoren van *M. graminicola* bescherming bieden tegen afbraak van schimmelcelwanden door enzymen die de plant produceert. Deze bevindingen laten zien dat LysM effectoren op verschillende manieren kunnen bijdragen aan de virulentie van plantpathogene schimmels.

De LysM effectoren Mg3LysM van *M. graminicola* en Ecp6 van *C. fulvum* lijken sterk op elkaar en hebben beide drie LysM domeinen. Maar terwijl Mg3LysM bescherming biedt tegen plantenzymen die de schimmelcelwand afbreken, heeft Ecp6 deze eigenschap niet. **Hoofdstuk 3** beschrijft het onderzoek naar de bijdrage van de LysM domeinen van Mg3LysM aan het beschermend vermogen van deze effector. Hiertoe zijn eiwitvarianten geanalyseerd waarin LysM domeinen van Mg3LysM zijn uitgewisseld met de corresponderende LysM domeinen van Ecp6. Zowel het eerste als het derde LysM domein van Mg3LysM zijn betrokken bij het bieden van bescherming tegen plantenzymen, terwijl het tweede LysM domein van Mg3LysM vervangen kan worden door het tweede LysM domein van Ecp6 zonder verlies van het beschermend vermogen.

LysM effectoren komen niet alleen voor in schimmels die planten via het blad infecteren, maar ook in bodempathogenen die hun gastheer binnendringen via de wortels. **Hoofdstuk 4** beschrijft het onderzoek naar LysM effectoren van de bodemschimmel *Verticillium dahliae* die de vaatbundels van zijn gastheer koloniseert. Van elf *V. dahliae* isolaten zijn de genoomsequenties beschikbaar en in elk isolaat komen vier LysM effectoren

voor die worden aangeduid als de 'kern' LysM effectoren. Geen van deze LysM effector genen komt echter tot expressie in *V. dahliae* tijdens infectie van tomaat en *Nicotiana benthamina*. Daarnaast leidt gerichte uitschakeling van de LysM effector genen niet tot verminderde virulentie van *V. dahliae* op deze gastheren. Deze waarnemingen suggereren dat de kern LysM effectoren van *V. dahliae* geen rol spelen tijdens infectie. Naast het deel van het genoom dat gelijk is voor alle elf *V. dahliae* isolaten, komen ook isolaat-specifieke genomische regio's voor. In een isolaat-specifieke regio van *V. dahliae* isolaat VdLs17 werd een extra LysM effector gen (*Vd2LysM*) gevonden dat afwezig is in de andere tien isolaten. Opmerkelijk is dat Vd2LysM wel bijdraagt aan virulentie van *V. dahliae* VdLs17. Net als eerder gekarakteriseerde LysM effectoren, blijkt ook Vd2LysM chitine te binden en chitine-geïnduceerde immuniteit te onderdrukken. Deze resulatateN suggereren dat Vd2LysM bijdraagt aan virulentie van VdLs17 door tijdens kolonisatie van de gastheer de chitine-geïnduceerde immuniteit te onderdrukken.

Tot dusver is aangetoond dat LysM effectoren bijdragen aan virulentie van verschillende plantpathogene schimmels. Niet-pathogene schimmelsoorten hebben echter ook LysM effector genen, wat suggereert dat LysM effectoren ook andere functies hebben waarmee ze bijdragen aan de biologie van schimmels buiten een gastheer. In **Hoofdstuk 5** wordt de hypothese onderzocht dat LysM effectoren een rol spelen in interacties tussen schimmels en andere micro-organismen, waaronder bacteriën. Dit kan ook relevant zijn voor plantpathogene schimmels die te maken hebben met andere micro-organismen tijdens de infectie. Om deze hypothese te kunnen toetsen is eerst voor verschillende bacteriesoorten bekeken of ze een effect hebben op schimmelgroei *in vitro*. Hiertoe is gebruik gemaakt van de schimmel *Trichoderma viride*, aangezien chitine in de celwand van deze schimmelsoort toegankelijk is tijdens groei *in vitro*. De resultaten van experimenten waarbij *T. viride* wordt blootgesteld aan bacteriën in aan- en afwezigheid van LysM effectoren, suggereren dat LysM effectoren een rol spelen bij de bescherming van schimmels tegen bacteriële concurrenten.

In **Hoofdstuk 6** worden de belangrijkste bevindingen van dit proefschrift besproken en wordt in het bijzonder ingegaan op de (potentiële) rol van LysM effectoren in schimmels met verschillende levensstijlen, inclusief pathogene en niet-pathogene schimmels.

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I'm happy that Andrea and Ronnie are my paranimfs. Ronnie, vanzelfsprekend ben je heel belangrijk geweest voor mijn project, check alleen al de talloze referenties naar 'de Jonge et al.'! Je had altijd tijd om te helpen en uitleg te geven over bioinformatische zaken. Daarnaast ben je erg aangenaam gezelschap en ik verheug me op je bezoek aan Zürich! Ik wens je alle succes en geluk in de toekomst, zowel professioneel als privé met Manon en de kleine. Andrea, my LysM partner, I'm very happy that you came to the lab. I remember being a little nervous to meet the new colleague that would come to work on the LysM project, but it turned out to be great to have someone like you to work with. It was very helpful and interesting to discuss work and work-related issues with you and also a lot of fun to share all kind of other actvities and experiences. It is an amazing coincidence that we will both start working in Zürich this spring; I'm looking forward to share this new adventure!

Of course the good time I had is not only thanks to the Verticillium-group but to many people in the department of Phytopathology. Sometimes I might not have felt like work-

ing, but to be at work was usually a pleasure because of all the fun and interesting chats in the lab, behind the computer, during coffee and lunch breaks or drinks. It was great to be part of such an international community, which definitely enriched the PhD experience. Many thanks to all of you!!

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Curriculum vitae

Anja was born in 1985 and grew up in Kostvlies, Drenthe. After attending high school Ubbo Emmius in Stadskanaal, she moved to Wageningen in 2003 to start a BSc in Biology. She got interested in the molecular biology of plants and chose the specialisation 'plant biology' for the MSc in Biology, also in Wageningen. Her first master thesis project dealt with *N*-glycosylation in plants and was carried out in the Laboratory of Plant Physiology under supervision of Dr Sander van der Krol. In January 2008 Anja started a research internship of six months in the group of Prof. Sheila McCormick (UCBerkeley/USDA, California). Here, she worked with Dr Yan Zhang on the role of small GTPases in pollen tube growth. Back in Wageningen, she performed another thesis project, in the Laboratory of Phytopathology and within the group of Dr Bart Thomma. This project aimed to investigate the role of the *Cladosporium fulvum* effector Ecp6 in pathogen virulence and was supervised by Dr Peter van Esse. After her MSc graduation in 2009 she started a PhD project in the same group, on the functional analysis of LysM effectors of plant pathogenic fungi, of which the results are described in this thesis. From April 2014, Anja will start as a post-doctoral researcher in the group of Dr Markus Künzler at the ETH Zürich (Switzerland) to study molecular mechanisms of fungal defense against antagonistic microbes.

Publications

Kombrink A., Thomma B.P.H.J. (2013). LysM effectors: secreted proteins supporting fungal life. *PLoS Pathogens* **9** e1003769.

Liebrand T.W.H.*, Kombrink A.*, Zhang Z., Sklenar J., Jones A.M.E., Robatzek S., Thomma B.P.H.J., Joosten M.H.A.J. (2013). Chaperones of the endoplasmic reticulum are required for Ve1-mediated resistance to Verticillium. *Molecular Plant Pathology* **15** 109-117.

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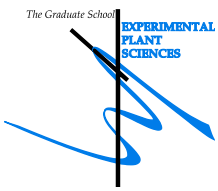
*equal contribution

Education Statement of the Graduate School
Experimental Plant Sciences



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1) Start-up phase	date
► **First presentation of your project**	
Recognition of the immune suppressor Ecp6 for broad-spectrum pathogen resistance in plants	Jun 08, 2009
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Book chapter: Heterologous production of fungal effectors in Pichia pastoris, In: Plant Fungal Pathogens:Methods and Procols (Melvin D. Bolton and Bart P.H.J. Thomma, eds), Vol 835, pp 209-217	2012
Review: The role of chitin detection in plant-pathogen interactions, Microbes and Infection 13 (2011), pp 1168-1176	2011
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Wageningen University	May 20, 2011
EPS PhD student day, University of Amsterdam	Nov 30, 2012
► **EPS theme symposia**	
EPS Theme 2: Interactions between Plants and Biotic Agents (WCS-day Utrecht)	Jan 15, 2010
EPS Theme 2: Interactions between Plants and Biotic Agents (WCS-day Amsterdam)	Feb 03, 2011
EPS Theme 2: Interactions between Plants and Biotic Agents (WCS-day Wageningen	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 22-23, 2013
► **Seminars (series), workshops and symposia**	
Nick Panopoulos: Playing the HRP: Evolution of Our Understanding of HRP Gene	Jan 11, 2010
Laurent Zimmerli: Functional characterization of two lectin receptor kinases involved in the Arabidopsis defense response against microbial pathogens	Jan 25, 2010
Naoto Shibuya: Chitin perception and signalling in rice and arabidopsis	Sep 09, 2010
Regine Kahmann: Effectors of the plant-pathogen fungus Ustilago maydis	Oct 29, 2010
Rosie Bradshaw: The genome of Dothistroma septosporum, a close relative of Cladosporium fulvum; what have we learnt so far?	Aug 04, 2011
Georgina May: Evolution of pathogen virulence in a community context	Jun 14, 2012
David Baulcombe: Rob Goldbach Memorial Lecture	Oct 10, 2012
Ralph Panstruga: Comparative pathogenomics of powdery mildew fungi: chasing the molecular secrets of obligate biotrophy and fungal pathogenesis	Dec 04, 2012
Howard Judelson: Molecular Insights into Spore Biology and Metabolism of Phytophthora infestans, the Potato Blight Pathogen	May 07, 2013
Rays Jiang: Integrative genomics of destructive pathogens from oomycetes to malaria parasites	May 07, 2013
Workshop: PSG immunology workshop	Aug 29, 2011
Joined meeting CBS-Phytopathology on Bioinformatics and Medical Mycology	Nov 12, 2010
2nd joined Wageningen-Marburg meeting	Jan 30-31, 2012
► **Seminar plus**	
► **International symposia and congresses**	
NCMLS Symposium 2009, New Frontiers in Pattern Recognition	Nov 05-06, 2009
ECFG11 Marburg, 2012	Mar 30-Apr 02, 2012
30th New Phytologist symposium; immunomodulation by plant-associated organisms	Sep 16-19, 2012
27th Fungal Genetics Conference, Asilomar	Mar 12-17, 2013
► **Presentations**	
Poster: Cologne, The conserved fungal effector Ecp6 inhibits chitin-triggered immunity	Apr 14, 2010
Oral: EPS Lunteren, LysM effectors of various fungal species contribute to pathogen virulence	Apr 05, 2011
Oral: Shandong Agricultural University, LysM effectors of various fungal species contribute to pathogen virulence	Oct 14, 2011
Oral: Autumn school Host-Microbe interactions, LysM effectors of various fungal species contribute to pathogen virulence	Nov 02, 2011
Oral: Wageningen-Marburg meeting, LysM effectors contribute to pathogen fitness through various mechanisms	Jan 30, 2012
Poster: ECFG, LysM effectors contribute to pathogen virulence in various manners	Mar 30, 2012
Oral: 27th Fungal Genetics Conference, The role of LysM effectors in fungal fitness	Mar 16, 2013
► **IAB interview**	
Meeting with a member of the International Advisory Board of the Graduate School	Nov 14, 2012
► **Excursions**	
Subtotal Scientific Exposure	*17.6 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
PhD Summerschool: Environmental signaling	Aug 24-26, 2009
PhD Autumnschool: Host-Microbe Interactomics	Nov 01-03, 2011
► **Journal club**	
Participation in literature discussion at PHP	2009-2013
► **Individual research training**	
Subtotal In-Depth Studies	*4.8 credits**

4) Personal development	date
► **Skill training courses**	
Course: Advanced Course Guide to Scientific Artwork	Nov 04-05, 2010
ExPectationS (EPS Career day)	Nov 18, 2011
NWO Pump your career - including workshop 'negotiation'	Oct 11, 2012
WGS course: Improve Your Writing	Nov 01,15,22-Dec 06, 2012
ExPectationS (EPS Career day)	Feb 01, 2013
Course: InDesign	May 08, 2013
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**32.9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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Cover: Fungal spore and growing hyphae, with in red a chitin molecule and on the right a representation of LysM1 of Ecp6.

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