Functional analysis of effectors from *Blumeria graminis* and their effect on the plant microbiome

Funktionell analys av effektorer från Blumeria graminis *och deras effect på växters microbiom*

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Abstract

With an expected population of 10 billion by the next century, agricultural production has to increase at the same pace to match such demand. One of the main obstacles to achieving this are the pathogenic microorganisms that reduce food quality and yields every year. Understanding the interactions between these microbes and their hosts is essential for shaping proper control strategies.

Phytopathogenic fungi utilize effectors, a class of small and diverse proteins, to interact with and supress the defences of the plants they infect. More recently, interest has been growing in the potential for alternative roles for these proteins, such as the interaction with other microbes in the environment and how they may be used to shape the local microbiome.

Here, eight proteins from a family of effector genes produced by *Blumeria graminis* f.sp. *tritici*, selected based RNA expression profiles showing increased expression during coinfections, were studied using heterologous protein production. Stable transformation of *Pichia pastoris* and transient expression in *Nicotiana benthamiana* through homologous recombination and Agroinfiltration respectively was used to perform assays testing the proteins for antimicrobial and phytotoxic activity. While no phytotoxic effects could be observed, three effectors displayed indications of antifungal activity and one in particular displayed both antifungal and antibacterial potential. Additional verification and optimization of the assays used here should be performed to improve the reliability of the results and potentially allow the protocols to be used for characterization of other effectors of obligate biotrophic plant pathogens.

***Keywords**:* **Antimicrobial** proteins, Heterologus protein expression, *Blumeria graminis* f.sp. *tritici, Pichia pastoris, Nicotiana benthamiana,* Effector proteins

Popular Science Summary

Earth's population is expected to grow to 10 billion by the next century. To sustain such a large number of people, agricultural production has to increase to match this future demand. One obstacle to this are the diseases that affect our agricultural crops and lead every year to big losses in the fields of farmers. Understanding the mechanisms behind the diseases, both how the pathogens infects its hosts and how plant immune systems responds, is crucial to preventing these loses and helping secure future food supplies. Recently, increasing attention was given to the role that the microbiome (*i.e.* the sum of all microbes living together with the plant) has to play in these mechanisms. Specifically, its interplay with the pathogen and to what extent they affect each other.

Here, three proteins from the plant pathogenic fungi *Blumeria graminis*, responsible for causing powdery mildew disease in grasses, were shown to display potential antibiotic activity against fungi and bacteria. To do this, the yeast *Pichia pastoris* and plant *Nicotiana benthamiana* were genetically modified to express the proteins of a gene family. These genes had previously been shown to be expressed by *B. graminis* when exposed to other, potentially competitive, fungi, and also in the field. While none of the proteins displayed toxicity to the plant, *N. benthamiana*, three of the proteins seem to inhibit the growth of fungi while one of them seem to inhibit both fungal and bacterial growth.

Because *B. graminis* is a biotrophic organism, it can only stay alive if the host is also alive (*i.e.* the pathogen does not kill the host). As such, it is hard to study it in a lab environment in artificial conditions (*e.g.* petri plates). The systems used here to study the effect of these proteins provide a framework for characterizing other proteins produced by *B. graminis*, along with other biotrophic fungi, in a manageable lab setting.

Characterizing proteins such as these can help us better understand the ecological role plant pathogens play in agricultural systems. Doing so might allow us to develop better management strategies as well as identify new targets in plant breeding with the potential to produce new varieties with better resistances. Additionally, antimicrobial proteins from pathogens may represent an untapped resource for future antibiotics.

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Abbreviations

AOX1	Alcohol Oxidase
BMGY	Buffered Glycerol-complex medium
BMMY	Buffered Methanol-complex medium
CA	Casamino acids
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
ER	Endoplasmic reticulum
GOI	Gene of interest
HR	Hypersensitive response
LB	Lysogeny Broth
MD	Minimal dextrose medium
ML	MD-2-related lipid recognition
MS	Murashige skoog
NLR	Nucleotide-binding leucine-rich-repeat
OD_{600}	Optical density (at wavelength 600 nm)
PAMP	Pathogen associated microbe pattern
PAOX1	Promoter for AOX1
PRR	Pattern recognition receptor
PTI	PAMP triggered immunity
ROS	Reactive oxygen species
rpm	Revolutions per minute
T-DNA	Transfer DNA
SAR	Systemic acquired resistance

1. Introduction

1.1. Plant immunity

With the earth's population expected to grow to 10 billion by the year 2100 (Raftery *et al.*, 2014), maintaining an increase in agricultural production to match that growth is a major challenge. Part of that challenge is containing and combating pathogens and pests which cause significant reductions in agricultural yields every year (Savary *et al.*, 2019).

An important component in controlling pests and pathogens is through breeding resistance into crops, thereby reducing the need for pesticides and other costly inputs otherwise required. However, in order for resistance breeding to be successful and durable, it is important to understand the nature of the interaction between pathogens and their hosts and its molecular underpinnings (Dodds and Rathjen, 2010). Furthermore, that interaction is also shaped by the environment, be it biotic, such as the presence of other microorganisms (Saunders *et al.*, 2012), or abiotic stress (Lolle *et al.*, 2020).

To defend themselves against pathogens, plants rely on two layers of immunity, PAMP triggered immunity (PTI) and effector triggered immunity (ETI). PAMP stands for pathogen-associated molecular patterns and refers to conserved motifs, associated with microbes that evolve slowly and are present in organisms across many different groups and species. Two well-known examples of this are chitin, part of the fungal cell wall and bacterial flagellin, used by bacteria for mobility (Schwessinger and Zipfel, 2008). PAMPs such as these can be detected by two different types of proteins in the plant: receptor-like kinases and receptor-like proteins which together are referred to as Pattern Recognition Receptors (PRRs), which are located at the membrane of the plant cells. (Ben Khaled *et al.*, 2015). These motifs activate signalling pathways which induces a series of responses in the cell, including spikes in the production of reactive oxygen species (ROS) and defence related proteins, increased expression of defence related genes and the hypersensitive response (HR) which ultimately kills the cell (Chisholm *et al.*, 2006, Bigeard *et al.*, 2015) Beyond these local immune responses, the detection of a pathogen by the host also leads to distal effects in the plant, such as Systemic

Acquired Resistance (SAR) which primes cells in other parts of the plant, making them less susceptible to infection (Mishina and Zeier, 2007). As such, the PTI contributes to the so-called non-host immunity of plants against the majority of potentially pathogenic microbes (Lo Presti *et al.*, 2015).

To avoid activating PTI, fungi have evolved effector proteins as a response. Effector proteins are commonly defined as small (<300 aa) secreted proteins, containing disulphide bridges (Lo Presti *et al.*, 2015). The secretion of these proteins is usually facilitated by a signal peptide (SP) at the N-terminal of the protein, which is cleaved once the protein leaves the cell (Rapoport, 2007). Once secreted, the effectors can be localized either to the apoplast or directly into the host cell (Win *et al.*, 2012). Considering their inherent diversity, effectors are proposed to fulfil a variety of biochemical functions but have mostly been shown to interfere with the host defences or otherwise increase host susceptibility to the pathogen (Franceschetti *et al.*, 2017). For example, the fungal pathogen *Cladosporium fulvum* produces an effector, which binds with ultrahigh affinity to chitin so as to outcompete the PRRs recognition by the host, thereby masking pathogen presence (De Jonge *et al.*, 2010). *Phytophtora infestans,* on the other hand, produces an effector which interferes with a protein that is important for the proper function of the signalling pathways mediated by PTI, thus interrupting the host response to the pathogens presence rather than masking it. (King *et al.*, 2014). As such, effectors induce susceptibility of the host plant to the pathogen, which is called Effector-triggered susceptibility (ETS) (Jones and Dangl, 2006), and therefore play a central role in the adaption of a pathogen to its host.

To counteract effectors, plants have evolved a class of intracellular receptor proteins belonging to the Nucleotide-binding leucine-rich repeats (NLRs) gene family. NLRs function to detect the presence of effectors, either by directly sensing their presence through direct physical binding (i.e. receptor-ligand model) , or indirectly by monitoring the targets of effectors and detecting modifications of these (Baggs *et al.*, 2017). The subsequent activation of NLRs produces a similar cellular response as PTI and shares many of the signalling pathways, leading to effector-triggered immunity (ETI) (Lu and Tsuda, 2021).

The presence of plant NLRs able to trigger ETI invariably leads to a selection pressure which favours pathogens that are able to evade ETI, either through gene loss (Huang *et al.*, 2014), down-regulated expression (Bourras *et al.*, 2018), or the evolution of novel effectors, capable of supressing ETI (Bourras *et al.*, 2015a), that once again establish ETS.

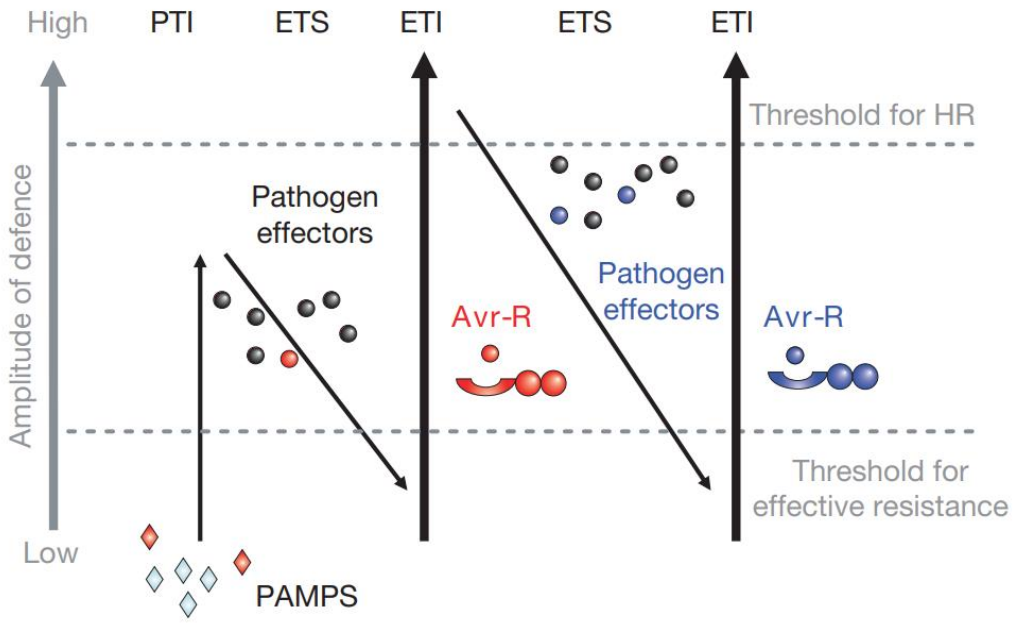

Figure 1. The zig-zag model conceptualizes the co-evolution of immunity and susceptibility between plants and pathogens (Adapted from Jones & Dangl, 2006).

This evolutionary relationship between a pathogen and its host is described by Jones and Dangl (2006) in their zig-zag model (Figure 1) which conceptualizes this 'oscillation' back and forth as host and pathogen co-evolve when new effectors and NLRs emerge to confer susceptibility or immunity, respectively.

1.2. Powdery mildew

Powdery mildew is a plant disease that affects many economically important crops around the world and is caused by ascomycete fungi of the order *Eryisphales* (H. Gwynne-Vaughan). On cereals, the causative agents are members of the species *Blumeria graminis* (DC.) and are, economically, some of the most damaging forms of powdery mildews *(*Dean *et al.*, 2012*)*. Notably, fungi causing powdery mildews are obligate biotrophs, *i.e.* they can only survive on a living host. Their growth is epiphytic, and on cereals, infections mainly occur on the leaves (Zhang *et al.*, 2005). When a conidium germinates, a hyphal structure called an appressorium is formed, which releases lytic enzymes that facilitates the penetration of the host cell wall. After about 12 hours, a penetration peg is formed which can penetrate the weakened cuticle and cell wall of the plant (Zhang *et al.*, 2005). Once inside the cell, the tip of the penetration peg eventually swells to form a haustorium, a feeding structure common in biotrophic fungi, which is used for nutrient uptake and the release of effectors (Polonio *et al.*, 2020). Following successful infection, secondary hyphae will branch out and can establish up to 50 additional haustoria. Over a period of

four days, a single colony can produce up to 200 000 new conidia, which are wind-dispersed, allowing for a rapid and wide spread (Zhang *et al.*, 2005).

While the species *B. graminis* infects grasses and cereals, individuals of the species are further divided into *formae speciales* (f.sp), each of which can only infect a single host, *e.g. Blumeria graminis* f.sp. *tritici (B.g. tritici)* can only infect wheat (*Triticum aesitivum* L.) while *B.g. secale* can only infect Rye (*Secale cereal* L.) and so on, making each *formae specialis* highly adapted to its host (Troch *et al.*, 2014).

Central to this adaption is the effector proteins produced by *B. graminis.* Specifically those released by the haustorium which suppress plant immunity (Polonio *et al.*, 2020). In a study by Praz *et al.* (2018), the authors used RNA sequencing to measure differences in gene expression between strains of *B.g. tritici*, *B.g. secale* as well as *B.g. triticale*, a recently evolved hybrid of the previous two, capable of infecting triticale, itself a hybrid of wheat and rye. They were able to show that differentially expressed effector genes were crucial in the adaption of *B.g. triticale* to the novel host, triticale. The central role that effectors play in host adaption, along with the narrow host range of *B. graminus* has been suggested to drive the diversification of the powdery mildew effector repertoire (Bourras *et al.*, 2018). *Blumeria graminis* effectors are generically defined as lacking sequence homology with functionally characterized proteins, excluding other powdery mildew effectors (Müller *et al.* (2019). A large number of effectors are maintained in the genome of cereal powdery mildews (Bourras *et al.*, 2018). A consequence of this is that these effectors are usually predicted through bioinformatics means. Müller *et al.* (2019) were able to identify 844 candidate secreted effector proteins (CSEPs) in *B.g. tritici*, roughly 10% of all its annotated genes. Since these effectors by definition do not share homology with functionally characterized proteins, the function of the majority of these remains unknown.

1.3. Pathogen-microbe interactions

While the study of effectors has mainly focused on the direct interaction with host proteins involved in immunity, plant pathogens also interact with other components of the environment they inhabit. As an obligate biotroph infecting the leaf, *B. graminis* is not only constrained by its single species host range, but also by the fact that it occupies the phyllosphere, which is the above ground parts of the plant. This ecological niche is relatively nutrient poor and exposes the pathogen to a harsh environment, which includes rain, UV-radiation and fluctuating temperatures (Delmotte *et al.*, 2009).

It is well-known that plants are capable of shaping the microbial community of the soil (Berendsen *et al.*, 2012). Particularly the rhizosphere, which is the soil immediately surrounding the roots, can be manipulated through secretion of

compounds that suppress or promote microorganisms (Berendsen *et al.*, 2018). These include organisms which display direct antagonism towards pathogens, such as *Clonostachys rosea* and *Trichoderma viride (*Antoniou *et al., 2017),* both of which are used as biocontrol agents against pathogens (Jensen *et al.*, 2002, John *et al.*, 2010), as well as organisms that can produce a similar response as systemic acquired resistance, priming the plant against infection before any pathogen has infected it (Compant *et al.*, 2005).

In contrast to the rhizosphere, the mechanisms used by plants to shape the microbial community in the phyllosphere, where *B. graminis* resides, is not as extensively studied (Vorholt, 2012). Still, the composition of the microbiome in the phyllosphere has been shown to be dependent on the species it belongs to. Redford *et al.* (2010) compared the bacterial communities of 10 different tree species and found that the host specie was the strongest predictor of its microbiome composition even across large distances. Similarly, Sapkota *et al.* (2015) found that in cereals, genotype was predictive of the microbiome composition, even on the cultivar level. Furthermore, Bodenhausen *et al.* (2014) found that changes to individual genes in *Arabidopsis thaliana* can shift microbiome composition in the phyllosphere, exemplifying causative mutations that shape the microbial composition above ground.

Since plant pathogens are under strong selective pressure not only from the host, but also from competition with the other microorganisms, Snelders *et al.* (2018) argued that plant pathogens likely shape their environment as well and that effector proteins most likely play a part in this. They also put this forward as an explanation for why the function of the majority of effectors remain unknown, since the search for their targets have been focused on the host. Considering this, and the inherent diversity of effector proteins, they suggest that effectors can be classified into three different groups depending on their target, (1) Plant-targeting, (2), Multifunctional and (3) Microbe-targeting. In support of this, the authors mention a paper by Kettles *et al.* (2018) where they characterize the effector Zt6 of the hemibiotroph *Zymoseptoria tritici*. Zt6 shows toxicity to both yeasts and bacteria as well as to the plant host, while itself remains unaffected by the effector, thereby exemplifying a multifunctional effector. Additionally Snelders *et al.* (2020) identified two effectors of the soil-borne pathogen *Verticillium dahliae,* VdAve1 and VdAMP2, both of which reduced the proliferation of several bacteria significantly and contributed to the virulence of the pathogen. The effectors did not inhibit any of the fungi tested or display any phytotoxicity and can therefore be considered an example of a purely microbe-targeting effector.

Beyond the opportunity to formulate better strategies to combat plant diseases, increased understanding of interactions between phytopathogens, their hosts and their environment might prove beneficial in another way. Snelders *et al.* (2020) also

suggest that effector proteins belonging to the multifunctional and microbe-targeting class could prove to be an untapped resource of novel antibiotics.

1.4. Effector characterization

Despite the large number of candidate effector genes identified across different pathogens and *B. graminis* in particular, characterizing their function remains a laborious task. Among the most relied upon means used for this purpose are methods that rely on disrupting or silencing the expression of the effector genes. This includes producing knockout or knockdown mutants through homologous recombination or RNAi methods (Dalio *et al.*, 2017). Host-induced gene silencing has for instance been used to identify effectors that contribute to virulence in *B. graminis* (Zhang *et al*., 2012). This method requires modification of the host, which is not necessarily an obstacle when characterizing effectors with targets in the plant. However, for effectors with targets outside the host *i.e.* multifunctional or microbe-targeting, such methods makes little sense. Unfortunately, no reliable protocols exist for transformation of *B. graminis* itself (Nowara *et al.*, 2010) and even if there were, the obligate biotrophy of *B. graminis* makes maintaining and testing any such transformed strains extremely challenging.

1.5. Heterologous protein production

Beyond the previously mentioned methods for functional characterization, a common approach is to use heterologous protein production. Especially, the tobacco relative *Nicotiana benthamiana* has been used widely to test whether effectors trigger the hyper-sensitive response (Goodin *et al.*, 2008). This is done by inserting the effector gene into *N. benthamiana* through *Agrobacterium tumefaciens* mediated transformation.

Another organism used for heterologous protein production is the yeast *Pichia pastoris*. *P. pastoris* is a methylotrophic yeast, *i.e.* it can metabolize methanol as a carbon source that has been used widely in the pharmaceutical industry for protein production. One of the main advantages of *P. pastoris* is the promoter for the alcohol oxidase 1 (AOX1) responsible for metabolizing methanol. In order to only express AOX1 when there is a lack of other carbon sources, the promoter is controlled through an induction/repression system, where expression is induced by the presence of methanol and but completely repressed by glucose (Turkanoglu Ozcelik *et al.*, 2019). This promoter can therefore be utilized in protein production by fusing this sequence in front of the gene of interest (GOI), which in essence provides an on/off switch.

1.6. Microbe targeting effectors in *Blumeria graminis f. sp. tritici*

In a study produced by Carol Kälin and collaborators (Masther Thesis, University of Zurich, -unpublished) an RNA sequencing of *B.g. tritici* was performed where expression profiles where compared from field meta samples, (*i.e.*, samples from wheat leaves in a natural environment, infected with *B.g. tritici* along with any other microbes present), and samples of wheat leaves co-infected with *B.g. tritici* and *Z. tritici* in a controlled laboratory environment. In doing this, Kälin and colleagues were able to identify a family of effector genes, hereafter referred to as the MD-2 effector family, which was significantly more expressed in the field than in the lab environment. This suggested that the proteins encoded by the genes in the MD-2 effector family provide a fitness benefit to the pathogen in field conditions and that some environmental factors had induced the up-regulation of their expression, unrelated to the host. In addition to this, Kälin and collaborators performed similar expression profiles comparing *B.g. tritici* on wheat, with or without co-infection by *Z. tritici*. In that case, the same gene family displayed increased expression in the samples co-infected with *Z. tritici*, further indicating that those effectors possibly confer fitness when competing with other microbes, possibly through some antimicrobial activity, similar to the effectors characterized by Kettles *et al.* (2018) and Snelders *et al.* (2020).

The MD-2 effector family is made up of eight genes, hereafter referred to as effector 1-8. Although it is the most highly expressed family when exposed to potential competitors, all the genes were not expressed equally. Four of the genes, effector 1, 3, 4 and 5, were among the 5% most expressed genes while the others were less expressed. With the exception of the signal peptide, the genes of the MD-2 effector family showed little conservation of the protein sequence. However, the pattern of amino acid properties indicated structural conservation and six of the eight proteins were found to contain an ML-domain (Pfam: PF02221) when searched against the NCBI conserved domain database (Marchler-Bauer *et al.*, 2017). The ML-domain has been shown to function as a lipid recognition domain, specifically of lipopolysaccharides (Inohara and Nuez, 2002). Furthermore, the predicted 3D structure of the three proteins most expressed in the field samples showed structural homology to a protein found in Japanese worker ants used in the detection sensory signals (Ishida *et al.*, 2014) as well as part of a human protein involved in the proper functioning of cholesterol transfer across membranes (Li *et al.*, 2016).

1.7. Objectives

The aim of this thesis is to evaluate the function of the putative effector proteins encoded by the genes in the MD-2 effector family in *B. graminis* f.sp. *tritici* specifically to test them for antibacterial, antifungal, and phytotoxic activity to determine if they can be considered multifunctional or microbe-targeting effectors (Snelders *et al.*, 2018).

To achieve this, heterologous gene expression is utilized to enable assays that isolate their function from the host. To test for potential phytotoxicity, *A. tumefaciens* is used to transiently transform *N. benthamiana* in order to express the genes of the MD-2 effector family. To measure the potential antimicrobial activity, *P. pastoris* is stably transformed using heterologous recombination to produce clones capable of producing the effector proteins upon induction.

2. Methods

2.1. Vector construction for *Pichia pastoris* transformation

To transform *P. pastoris*, pPIC9H (Liu *et al.*, 2003) was used as an expression vector. The genes of the *MD-2* family of effectors were optimized for expression in *P. pastoris* using the IDT Codon Optimization Tool (https://eu.idtdna.com/CodonOpt). Signal peptides were removed beforehand using SignalP 5.0 (Almagro Armenteros *et al.*, 2019). Optimized sequences were subsequently cloned *in silco* into pPIC9H using plasmid editors Snapgene or ApE to make sure no unintended restriction sites were introduced. Gene synthesis of the cloning vector pUC57 containing each of the optimized sequences flanked by restriction sites EcoRI and SnaBI were ordered from General Biosystems (Durham, NC, USA).

Initially, pUC57s containing the GOIs and pPIC9H were propagated in *E. coli* and screened for using ampicillin. Plasmids were extracted using GeneJet Plasmid Miniprep kit® (Thermo Scientific™). DNA concentrations were determined using NanoDrop® Spectrophotometer. Both plasmids were digested using restriction enzymes SnaBI and EcoRI. GOI fragments from pUC57 were separated by gel electrophoresis and purified using Silica Bead DNA Gel Extraction kit® (Thermo Scientific™). Digested pPIC9H was dephosphorylated to prevent relegation of the fragments. The T4 ligase was used to fuse the GOI with pPIC9H. The ligation reaction mix was used to transform One Shot® TOP10 Chemically Competent *E. coli*. Restriction digest using BamHI and gel electrophoresis was used to validate successful integration into pPIC9H.

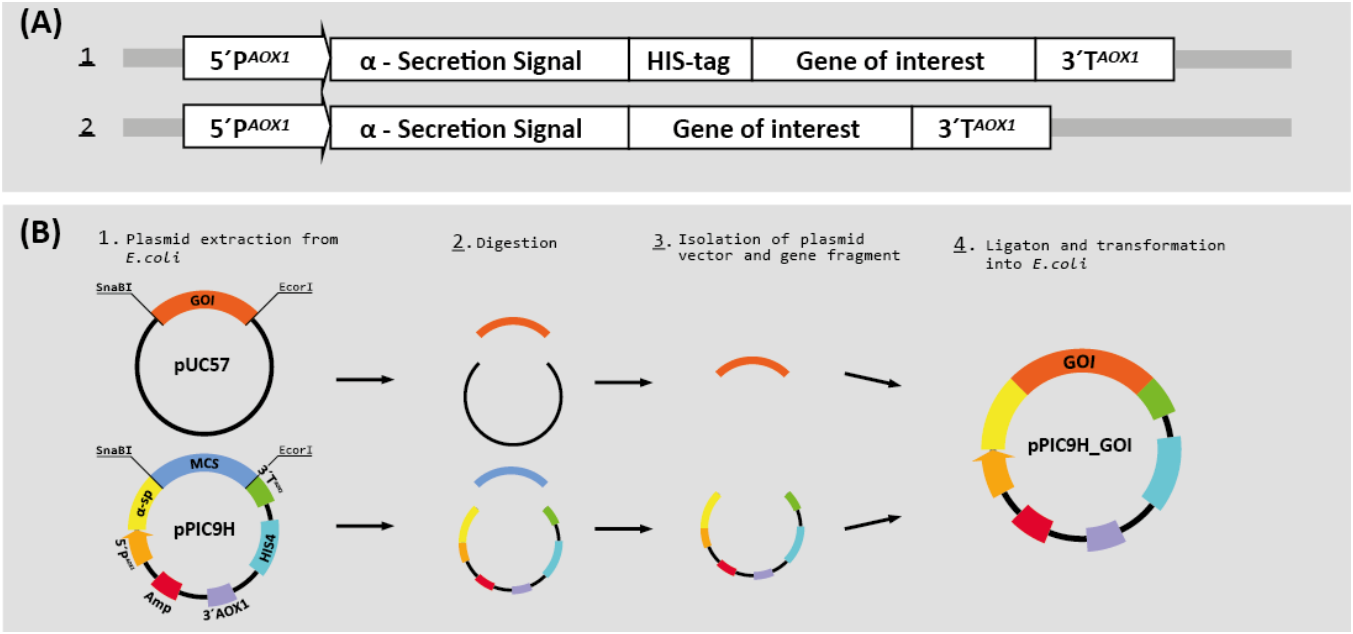

Figure 2. **(A)** Schematic of expression cassettes produced for expression vector pPIC9H. 5´PAOX1 is the methanol induced promotor associated with the *Aox1* gene which drive the expression of the cloned gene. Alpha-factor secretion signal is a signal peptide used to deliver the protein to the extracellular matrix. The His-tag is used for tagging and isolation of the mature protein. The 3´*Taox1* is the terminator sequence which halts the transcription. **(B)** Workflow of the vector construction. Step 1: Plasmids are multiplied in *E. coli* and extracted. Step 2: Both plasmids are digested using EcoRI and SnaBI. Step 3: pUC57 was separated from the GOI through gel-electrophoresis and the gel band containing the GOI was excised and purified. The digested pPIC9H was dephosphorylated to prevent self-ligation. Step 4: The GOI fragment and pPIC9 were ligated together. The product of that reaction was subsequently transformed in to *E. coli* and plated on selective media. To validate that the *E. coli* transformants carried the desired expression vector, plasmids were extracted and digested with EcoRI and BamHI.and analysed with gel-electrophoresis for correct fragment sizes.

2.2. Vector construction for *Nicotiana benthamiana* transformation

As with *P. pastoris* expression vectors, *MD-2* gene sequences were optimized for *N. benthamiana* expression using IDT Codon Optimization Tool (https://eu.idtdna.com/CodonOpt). SignalP 5.0 (Almagro Armenteros *et al.*, 2019) was used to remove the signal peptide. To facilitate use of Gateway cloning for vector construction, *attL* cloning sites were added to the flanking regions of the optimized sequences. pUC57 constructs containing the optimized gene sequences along with flanking *attL* sites were ordered from General Biosystems (Durham, NC, USA). The vector pIPKb004 (Himmelbach *et al.*, 2007) was used as the expression vector into which the *MD-2* gene were to be cloned. pIPKb004 carries the toxin producing gene *ccdB* and has to be maintained in a tolerant strain of *E. coli*. The

gene constructs were subsequently cloned into pIPKb004 using the Gateway LR Clonase reaction according to manufacturer's protocol. Resulting constructs were transformed into One Shot® TOP10 Chemically Competent *E. coli* and screened for on lysogeny broth (LB) agar with spectinomycin.

2.3. *Pichia pastoris* transformation

The pPIC9H vector constructs described above were used to transform *P. pastoris* strain GS115 according to the LiCl transformation (Figure 3;2) protocol outlined in the Invitrogen™ Multi-Copy Pichia Expression Kit manual. The pPIC9H expression vector for seven of the eight effectors as well as the empty pPIC9H (to be used as control) were linearized using restriction enzyme StuI to promote insertion at the HIS4 locus (Figure 3;1). Due to the presence of restriction sites in the *effector 2* gene sequence, its vector remained undigested. Transformants were screened on Minimal Dextrose Media (MD) agar (Figure 3;3) and to validate successful integration into the *P. pastoris* genome, direct PCR screening was performed on single colonies as described in Invitrogen™ Multi-Copy *Pichia* Expression Kit manual. Colonies were simultaneously propagated in liquid MD for propagation and preparation of glycerol stocks to be stored at -80°C.

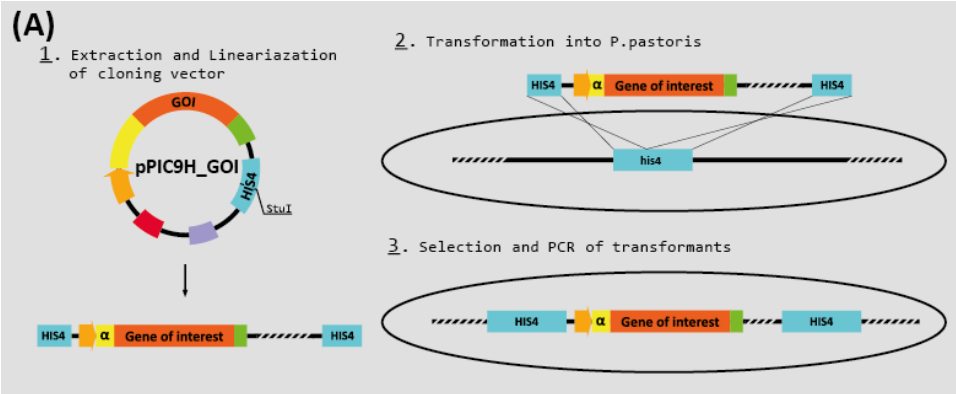

Figure 3. Workflow for transformation of *P. pastoris*. Step 1. Expression vectors containing the GOI were extracted from *E. coli* and linearized using restriction enzyme StuI. This was done to promote insertion at the his4 locus. Step 2: Transformation of vector DNA into *P. pastoris* strain GS115 using LiCl. Step 3: Transformation solution is plated on histidine-deficient media and incubated for 3 days.

2.4. *Nicotiana benthamiana* transformation and phytotoxicity assays

Agrobacterium tumefaciens infiltration was used for transient transformation of *N. benthamiana*. pIPKb004 constructs described previously were extracted from *E. coli* using GeneJET Plasmid Miniprep Kit (Thermo Scientific) and subsequently transformed into *A. tumefaciens* strain GV3101 made chemically competent using $CaCl^2$.

For cultivation of *N. benthamiana,* seeds sterilized in 10% bleach were placed on half-strength MS-agar plates supplemented with 1% sucrose and incubated for three weeks at a 16/8 day-night cycle. After incubation, seedlings were transferred to pots and grown in the same conditions for an additional three weeks.

Preparations of *A. tumefaciens* cultures and infiltrations were made as described in Ma *et al.* (2012) and Bourras *et al.* (2015b). In addition to the eight pIPKb0004 constructs containing the *MD-2* genes, pIPKb004 vectors carrying the *Zymoseptoria tritici* effector *AvrStb6* (Ziming *et al.*, 2017) and the empty expression cassette were used as positive and negative controls respectively.

2.5. Antimicrobial assays

2.5.1. Day 1

For each effector, four different *P. pastoris* clones, as well as four clones carrying the empty expression cassette, confirmed through Direct PCR screening as described above, were revived from glycerol stocks and incubated overnight in falcon tubes with five ml of buffered glycerol-complex media (BMGY) at 30°C and 180 revolutions per minute (rpm) shaking. BMGY does not contain methanol and thus the AOX1 promoter (PAOX1) will therefore not be induced to produce the effector gene.

2.5.2. Day 2

100-200 µl of *P. pastoris* cultures from the previous day were used as inoculum for 5 ml of (BMGY) in fresh falcon tubes and incubated overnight at 30°C and 180 rpm.

E. coli strain One Shot® TOP10 was revived from glycerol and incubated overnight in falcon tubes with five ml of liquid LB at 37°C and 180 rpm.

2.5.3. Day 3

On day 3, *P. pastoris* cultures were normalized to an OD_{600} value of one. Five ml of normalized cultures were then centrifuged at 3000 rpm for five minutes, and the supernatant discarded. The cell pellets were resuspended in five ml of the induction media, buffered methanol-complex medium (BMMY), and incubated for 18 hours at 30°C and 180 rpm. In fresh falcon tubes, 200 µl of *E. coli* culture from the previous day was used to inoculate 5 ml LB and cultures were incubated overnight at 37°C and 180 rpm.

2.5.4. Day 4

After 18 hours of induction, the OD^{600} was measured in order to observe the yeast growth rate. After this, the culture supernatants were separated from the cells by centrifugation at 3000 rpm for five minutes and carefully transferred without disturbing the cell pellet to a two ml centrifugal tube. From this point, the supernatants were kept on ice to prevent protein degradation and subsequently centrifuged at 17 000 rpm for five minutes and transferred to fresh 2 ml tubes to ensure no *P. pastoris* cells remained in the solution.

E. coli was pelleted through centrifugation at 3000 rpm for five minutes and the supernatant was discarded. The cell pellet was then resuspended to an OD^{600} of 0.1 in $1/10^{th}$ LB. A 96 well plate with flat bottom 300 µl wells was used to test the antibacterial activity of the cell culture supernatants. To each well, 100 µl of *E. coli* along with 100 µl of the *P. pastoris* culture supernatant was added. Each clone was placed on one row, meaning 8 replicates per effector. 100 µl BMMY and 100 µl $1/10^{th}$ LB was used as blanks. The 96 well plate was then incubated at 37°C and 180 rpm shaking. OD^{600} measurements were taken at 1-hour intervals for 5 hours.

3. Results

3.1. Vector construction for *Pichia pastoris* transformation

Figure 2.B shows the workflow of the vector construction. Using restriction enzymes SnaBI and EcoRI, gene fragments of *MD-2* effector family were extracted and isolated from the pUC57 cloning vector (Figure 4).

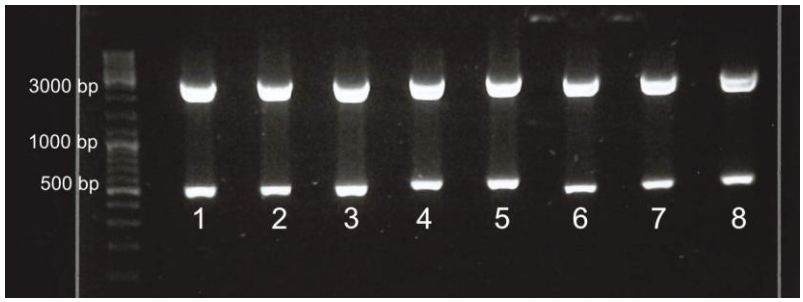

Figure 4. Gene fragments from digested pUC57 separated through gel electrophoresis. Smaller fragment represents the MD-2 candidate effector genes, 1 through 8.

Two variants for each effector were created, one with and one without a His-tag for potential protein isolation. Isolated gene fragments of the MD-2 effectors were ligated with pPIC9H and transformed into *E. coli* for propagation. Clones growing on selective media were confirmed to carry desired expression vector through gel electrophoresis of extracted plasmids digested using restriction enzymes EcoRI and BamHI. At least one clone for each expression cassette were validated (Figure 5). Effector 2 has two bands due to an additional restriction site for BamHI inside the gene.

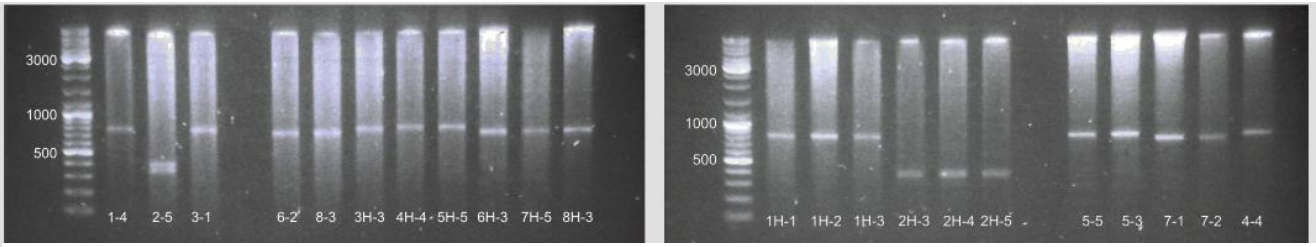

Figure 5. Gel-electrophoresis of expression vectors containing the GOI, digested with EcorI and BamHI. First digit denotes which effector. Expected size: Effector 1: 767, Effector 2: 408/359, Effector 3: 770, Effector 4: 797, Effector 5: 797, Effector 6: 737, *Effector 7: 740, Effector 8: 746.*

3.2. Vector construction for *Nicotiana benthamiana* transformation

Constructs for all eight MD-2 effector genes were successfully constructed using Gateway cloning and screened for using spectinomycin as previously described in Bourras et al. (2018).

3.3. *Pichia pastoris* transformation

In order to characterize the candidate effector proteins of the *MD-2* gene family, transformation of *P. pastoris* using homologous recombination was used to enable heterologous protein production. Clones were screened through direct PCR (Figure 6). Of the nine transformations conducted, (including all eight genes of the *MD-2* family and the empty expression vector) all generated viable clones except effector 8 (Table 1), which also failed on repeated attempts to generated transformants.

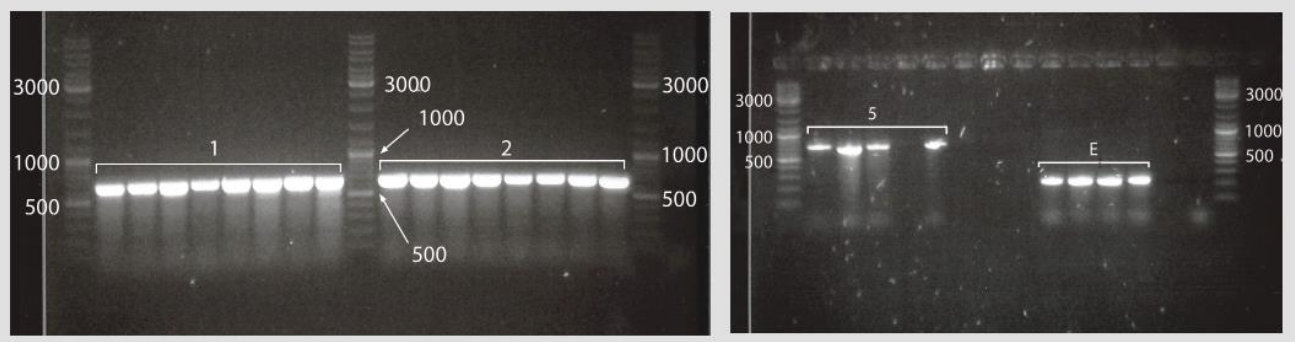

Figure 6. Gel-electrophoresis of Direct PCR screening product from transformed P. pastoris clones. E denotes clone transformed with the empty expression vector. Expected sizes 1:678 bp, 2: 678 bp, 5: 708 bp & E: 279 bp

Table 1 Number of successful P. pastoris *transformants obtained per* MD-2 *gene.*

Gene	*Number of successful clones*
Effector 1	*14*
Effector 2	*14*
Effector 3	*4*
Effector 4	*4*
Effector 5	*4*
Effector 6	*14*
Effector 7	*14*
Effector 8	*0*
Empty Cassette	*4*

3.4. *Nicotiana benthamiana* transformation and phytotoxicity assay

To investigate whether any of the *MD-2* effectors have a phytotoxic effect on plants, transient transformation via *A. tumefaciens* infiltration of *N. benthamiana* was conducted using vectors constructed as described above. Despite using both a positive and negative control, no effect could be detected on the *N. benthamiana* leaves (Figure 7).

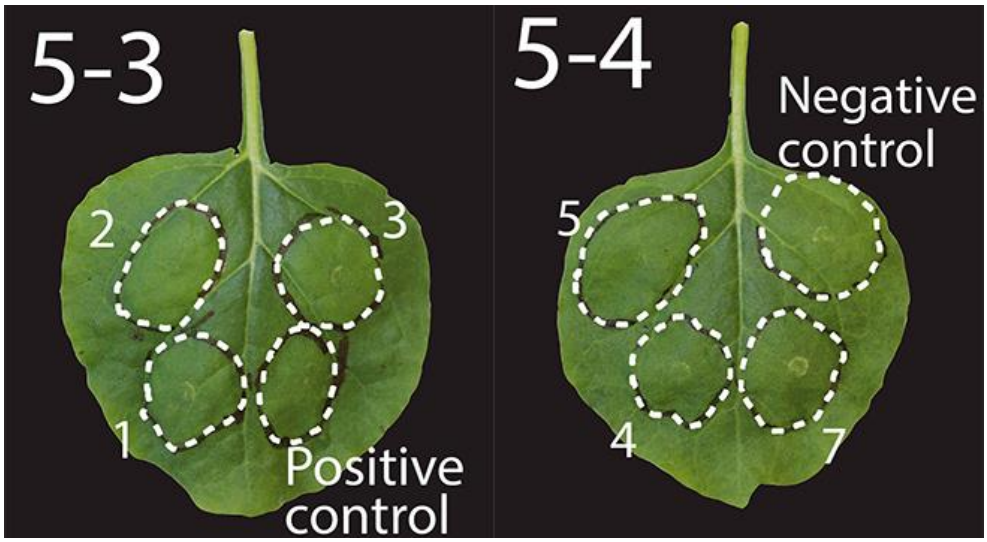

Figure 7. Infiltration of *N. benthamiana* with effectors 1,2,3,4,5,7, positive and negative control. No effect visible. Results for all infiltrations can be seen in Figure S2.

3.5. Antimicrobial assays

The antimicrobial assays were used to test the *MD-2* effectors potential for antifungal and antibacterial activity. The antifungal assay (2.5.4.) was conducted by inducing *P. pastoris* transformants to produce the effector proteins overnight, and comparing their growth to that of the control (transformants carrying the empty expression cassette) to detect inhibitions of growth. Measurement readings were only taken at the start (OD_{600} 1.0) and endpoint. Some of the effectors show indications of growth inhibition (Figure 8.A).

To test the antibacterial activity, culture supernatant from the previous assay was harvested and used as substrate for *E. coli* growth over five hours. Initial experiments indicated some inhibition from effector 1 and 2 (Figure 8.B). The assay

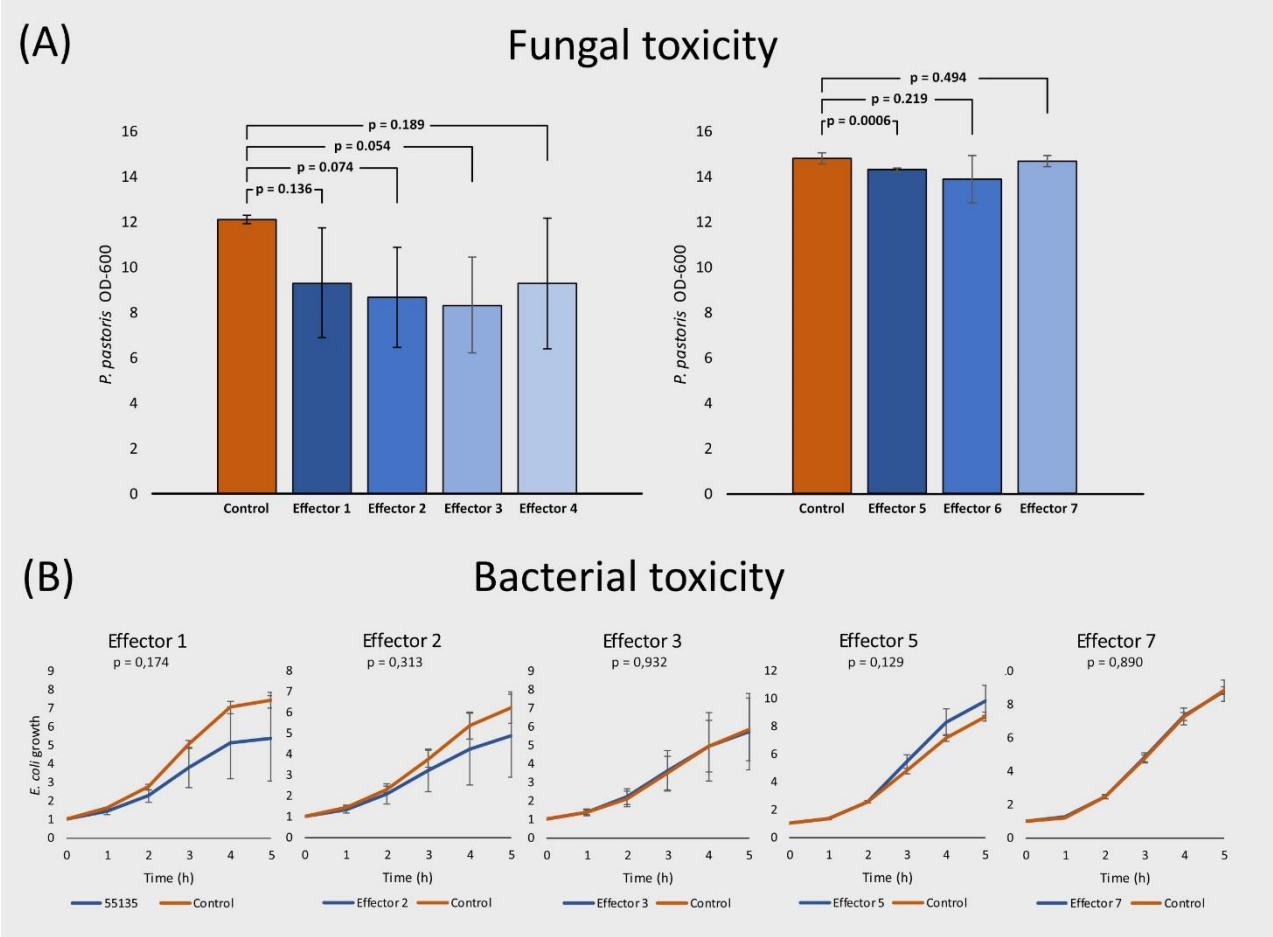

Figure 8 (A) Results from antifungal assay. OD_{600} of *P. pastoris* following 18 hours of induced protein production for the 7 of putative effector proteins. Control is the *P. pastoris* transformants carrying the empty expression cassette. Error bars indicate standard deviation. Effectors 1,2 and 3 exhibit tendency of inhibitory effect on growth, though results are not statistically significant. **(B)** Results from antibacterial assay. Growth of *E.coli* in cell culture supernatant of *P. pastoris* transformants carrying effector 1,2,3,5 and 7. Error bars indicate standard deviation. As in antifungal assay, effector 1 and 2 display tendency of growth inhibition, although not statistically significant.

was therefore repeated for those two (see Figure S4). Further testing with a new set of four clones carrying effector 2 reinforced the finding that the culture supernatant from those clones inhibited the growth of *E. coli.*

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4. Discussion

4.1. Effector characterization

Pathogenic fungal effectors are a diverse group of small secreted proteins that have been studied extensively in the context of plant-pathogen interactions, such as in their role of supressing plant immunity or extracting host resources (Jones and Dangl, 2006, Lo Presti *et al.*, 2015, Franceschetti *et al.*, 2017). More recently, attention has shifted towards alternative modes of action for these genes, specifically competition with and shaping of the community of microbes in the environment (Snelders *et al.*, 2018, Snelders *et al.*, 2020, Kettles *et al.*, 2018). Characterizing effectors with such non-traditional functions not only offers an opportunity for discovering novel antibiotics but can also offer insights into pathogen-microbe interactions that can inform management strategies in agriculture (Snelders *et al.*, 2020, Rovenich *et al.*, 2014).

Blumeria graminis f. sp. *tritic*i is a fungal pathogen of wheat (*Triticum aesitivum*). *B.g. tritici* maintains over 700 putative effector genes (Menardo *et al.*, 2017, Pedersen *et al.*, 2012), and as with other obligate biotrophs, a large majority of these proteins remain uncharacterized (Lo Presti *et al.*, 2015). The goal of this thesis was to characterize the eight proteins produced by the *MD-2* gene family of *B.g. tritici*. Kälin (Unpublised) previously showed that several of the effectors were highly expressed when *B.g. tritici* was exposed to environments with competing microorganisms, suggesting a fitness benefit in those circumstances. Heterologous protein expression in *P. pastoris* and *N. benthamiana* was used to produce the effector proteins in order to utilize them in assays to test them for antimicrobial and phytotoxic activity.

4.1.1. Phytotoxicity assay

Considering *B. graminis'* obligate biotrophic lifestyle, the expectation was for the MD-2 proteins to not have any phytotoxic activity. Since no effect could be observed for the positive control (Figure 7), the lack of effect displayed by the rest of the effectors (Figure S2) cannot be used to rule out phytotoxicity.

4.1.2. Antifungal assays

To test the effectors for antifungal activity, the transformed *P. pastoris* clones were assayed for self-toxicity (2.5.3). If any of the *P. pastoris* transformants were self-inhibited by the induction to express the effector genes, this would suggest a general antifungal activity. The results from the antifungal assay indicated some effect for several of the effectors (Figure 8.A), especially effector 1,2 and 3, with effector 2 displaying a trend over several replications of the assay (Figure 3S). Though the sample size is too small to draw definitive conclusions, the results motivate further investigation.

Kälin (Unpublished) showed that effectors from the MD-2 effector gene family were upregulated upon co-infection of wheat with *B.g. tritici* and *Z. tritici* in lab conditions when compared to infection with only *B.g. tritici*. It should be noted that effector 2 is not among the most highly expressed of the MD-2 effectors. However, since *P. pastoris* and *B.g. tritici* have two different natural environments, the soil and the leaf respectively, the inhibition of *P. pastoris* by the MD-2 effector proteins may suggest a broad antifungal activity for effector 1, 2 and 3. However, that does not exclude the prospect that the rest of the MD-2 effectors are antifungal. It is possible that the effectors are adapted to selectively target the microorganisms that occupies the same niche. For example, the effector VdAve1 of *Verticilium dahliae* was shown to have a strong selective antibacterial activity (Snelders *et al.*, 2020). Considering the upregulation caused upon coinfection with *Z. tritici*, it is possible that their activity is limited to other foliar microbes. It would therefore be of interest to test the MD-2 effectors activity against such organisms, *e.g. Z. tritici*.

Beyond the increased replicates which may lead to higher statistical confidence, the assay may be improved by adding a wash step before the induction media is added (2.5.3). The regulation of the PAOX1 in *P. pastoris* is tightly controlled, and in the presence of carbon sources other than methanol is almost completely repressed, even if methanol is present (Vogl and Glieder, 2013). Although centrifugation is used to separate the glucose media (BMGY) from the yeast cells before adding the methanol media (BMMY), any residual glucose might interfere with the induction before it is metabolized. If varying levels of the glucose is left, this could introduce variation between replicates and treatments.

4.1.3. Antibacterial assays

To test the antibacterial activity of the effectors, an assay was performed in which *P. pastoris* transformants were induced to produce the effector proteins in liquid media, after which the culture supernatant was used as a substrate for *E. coli* growth (2.5.4.). By measuring OD_{600} every hour, the growth rate of *E. coli* was determined. Of the seven effectors successfully transformed into P. pastoris, initially effector 1 and 2 showed the most promise as candidates for having antibacterial activity (Figure 8.B). After repeating the assay for those two effectors, the results for

effector 1 did not replicate and effector 2 became the focus of the assays (Figure S4). The culture supernatant from transformants carrying effector 2 did consistently display some inhibition of bacterial growth, even when the assay was replicated using different sets of clones (Figure S4).

As described in 4.1.1., it is possible that the activity of the effectors are adapted to microbes in its environment. Like *P. pastoris, E coli* is not a natural competitor and those effectors not inhibiting growth may be selectively toxic, similar to VdAve1 (Snelders *et al.,* 2020). Besides selectivity, if the MD-2 effector proteins actually are antimicrobial, there are several factors that might interfere with their function, even if expressed and secreted properly. It is possible that the culture supernatant is not the ideal environment for protein activity. The natural environment of *B.g. tritici* is the phyllosphere of wheat. Felle *et al.* (2004) measured the apoplastic pH of the stomata on wheat leaves during the initial stages of infection by *B.g. hordei* and measured the pH to between 5.0 and 5.5 over 24 hours. While there might be significant differences between locations on the leaf and time past infection, it is still of note that the media used in this assay was buffered to a pH of 6.0. Although not a drastic difference, the effectors might be tested using unbuffered media instead. This would acidify the supernatant due to *P. pastoris* natural secretome, possibly providing a more optimal pH for the proteins activity.

To improve the reliability of the assay, validation of insertions and protein production of the transformants should be considered. Due to time constraints, confirmation of successful insertion events was only conducted through Direct PCR screening (Figure 6). One of the features of the LiCl transformation protocol used here is the possibility of creating transformants which carry multiple copies of the expression cassette (Figure 2.A). However, the screening method used here does not allow for distinction of copy number variations. Therefore, some clones may produce more of the protein than others, introducing additional variation in the results. Sequencing of transformants would be an alternative to make the results more reliable. Western blotting could also be used to compare protein production between clones.

While methods such as western blotting would have shed light on the amount of protein produced by transformants, there are a number of obstacles that can interfere with heterologous protein production in *P. pastoris.* These include natural proteases that can be deactivated by adjusting pH (Cregg *et al.*, 1993) and endoplasmic reticulum (ER) stress alleviated through incubation temperature (Dragosits *et al.*, 2009, Zhong *et al.*, 2014), casamino-acid (CA) supplementation (Kaushik *et al.*, 2016) and alternative signal peptides (Barrero *et al.*, 2018). Even if the MD-2 effectors were expressed properly here, implementing such measures may be advisable to provide clearer results.

4.2. Outlook

The results gained from these assays provide an encouraging basis for further investigation into the MD-2 effector gene family and their potential role in manipulating the microbial ecology of its host. Especially the potential of effector 2 to inhibit bacterial growth. Further testing should focus on validating and optimizing protein production. Doing this would also improve confidence in replications of the assay, especially if used to test the MD-2 proteins against other organisms, in order to evaluate them for potential specificity to adapted antagonists or competitors.

Beyond the scope of the MD-2 effector family, the protocols used here provide a methodology for characterizing effectors proteins from other obligate biotrophic fungi, especially those that interact with targets outside the host. By extension this could shed light on mechanisms determining aggressiveness in plant pathogenic fungi. Such information may be useful for population ecology of pathogens and their ability to overcome natural competitors contributing to plant survivability and perhaps even future biocontrol agents.

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5. Supplementary material

5.1. Supplementary figures

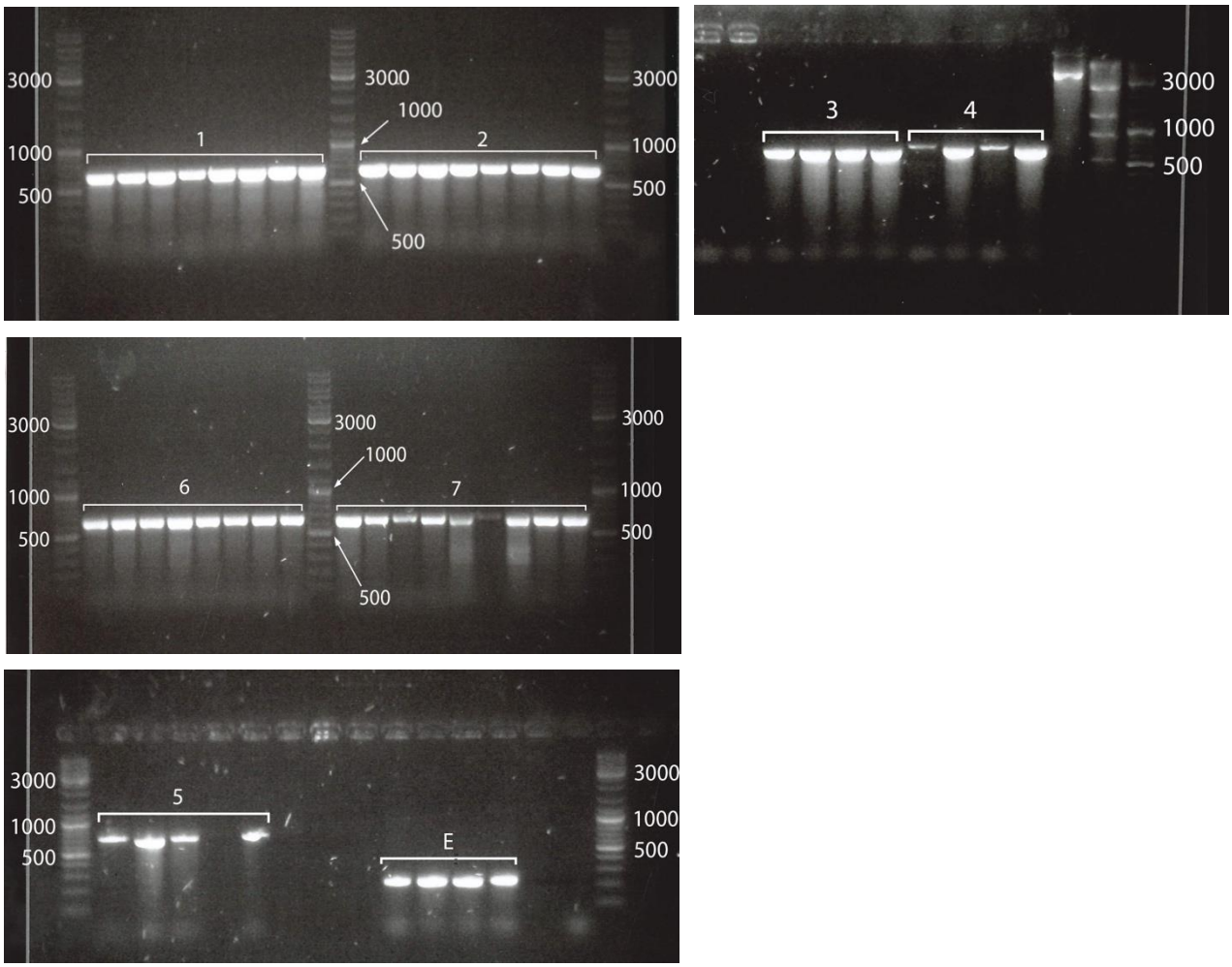
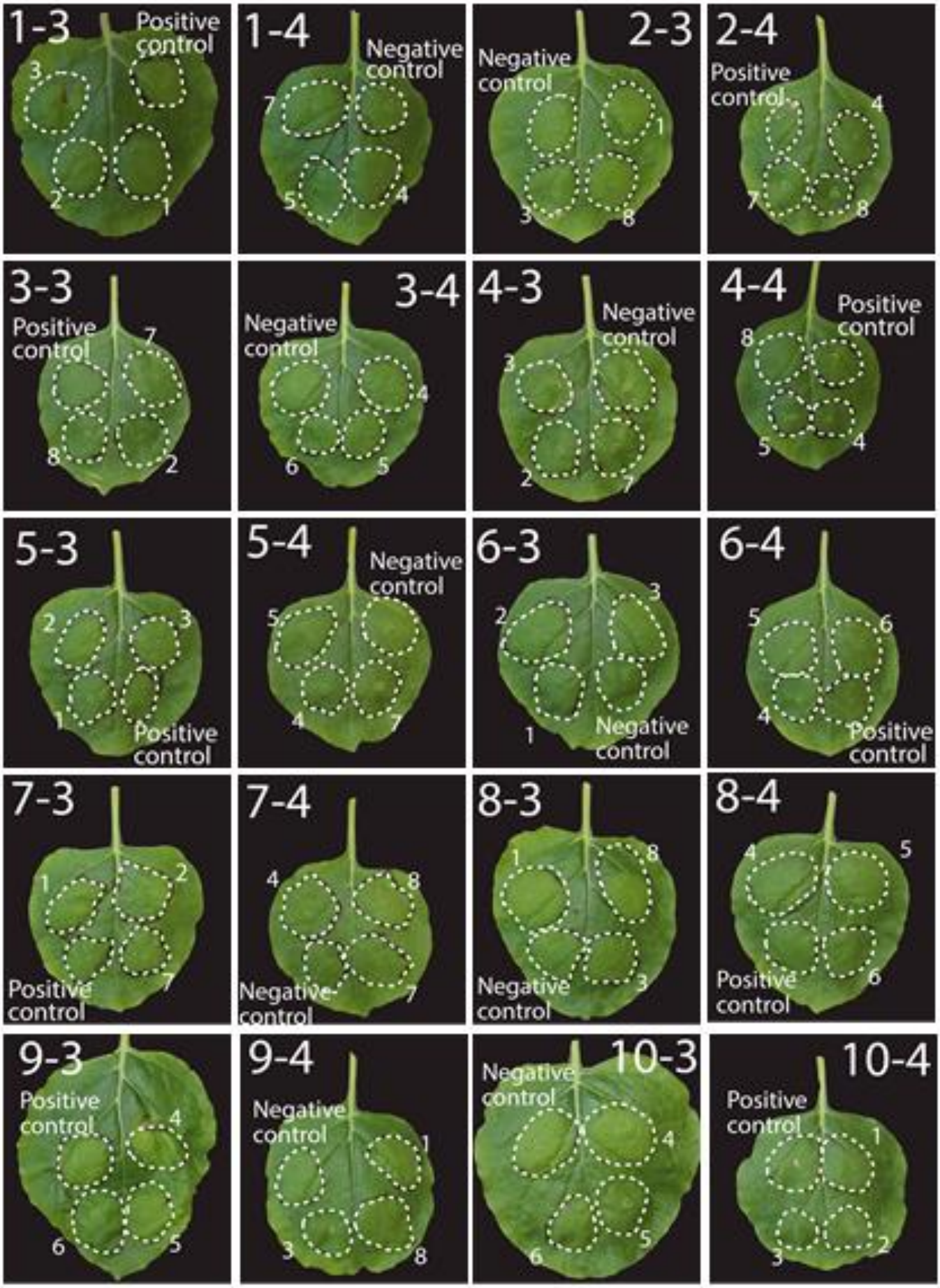


Figure S1. Gel electrophoresis of Direct PCR screening of *Pichia Pastoris* transformants carrying MD-2 effectors 1-8. 'E' denotes empty expression cassette. Expected sizes; 1:678 bp, 2:678 bp, 3:681 bp, 4:708 bp, 5:708 bp, 6:648 bp, 7:651 bp, E:229 bp.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Positive control
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2
1
1-4
Negative control
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5
4
2-3
Negative control
1
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8
2-4
Positive control
4
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Positive control
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Negative control
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Negative control
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Positive control
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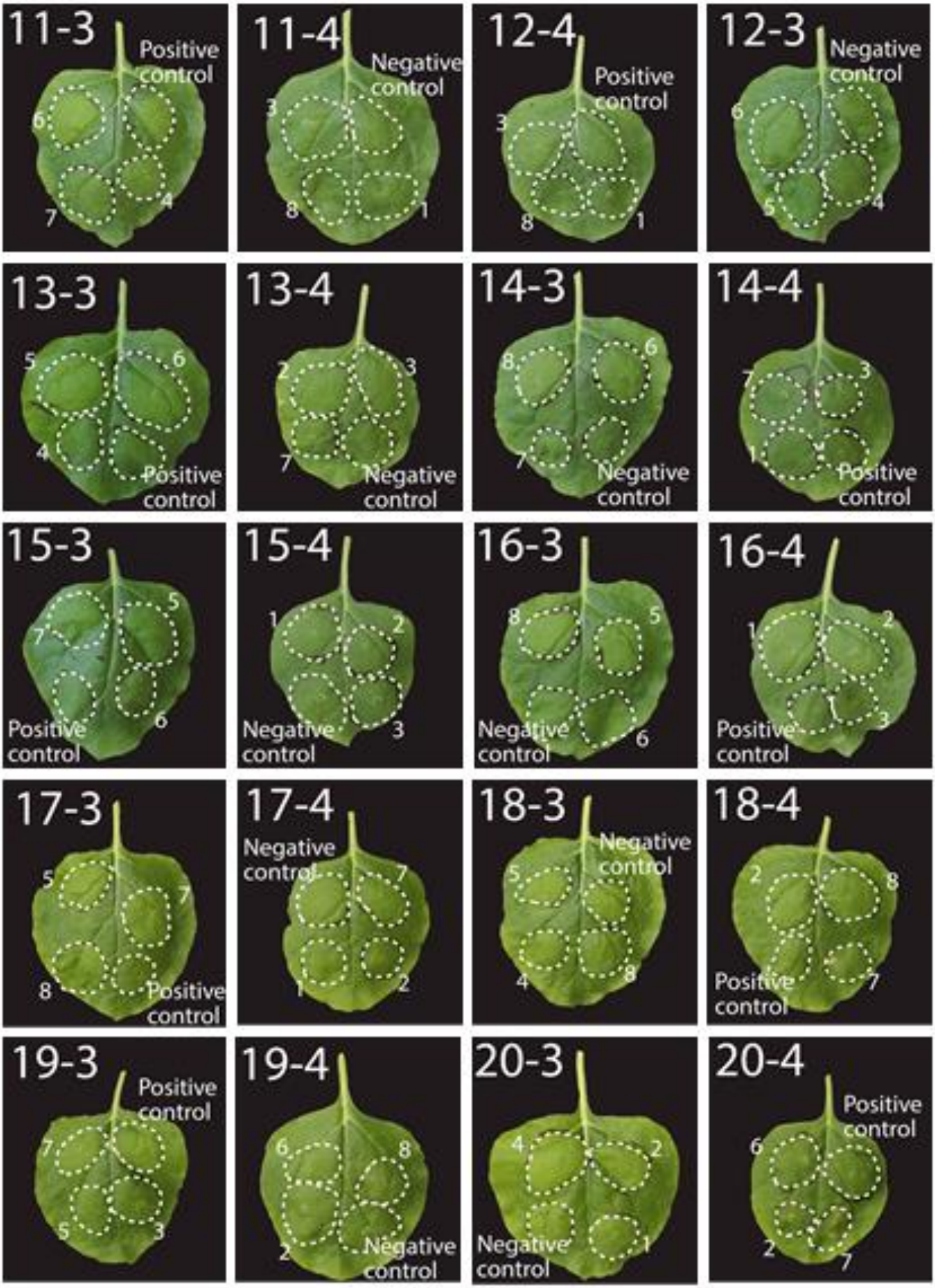

Figure S2. Agroinfiltrations of *Nicotiana benthamiana* on the 3rd and 4th leaves with Ti-plasmid pIPKb004 carrying MD-2 effector genes 1-8 as well as AvrStb6 as positive control and the empty expression cassette as negative control. Striped lines demarcate extent of infiltration,

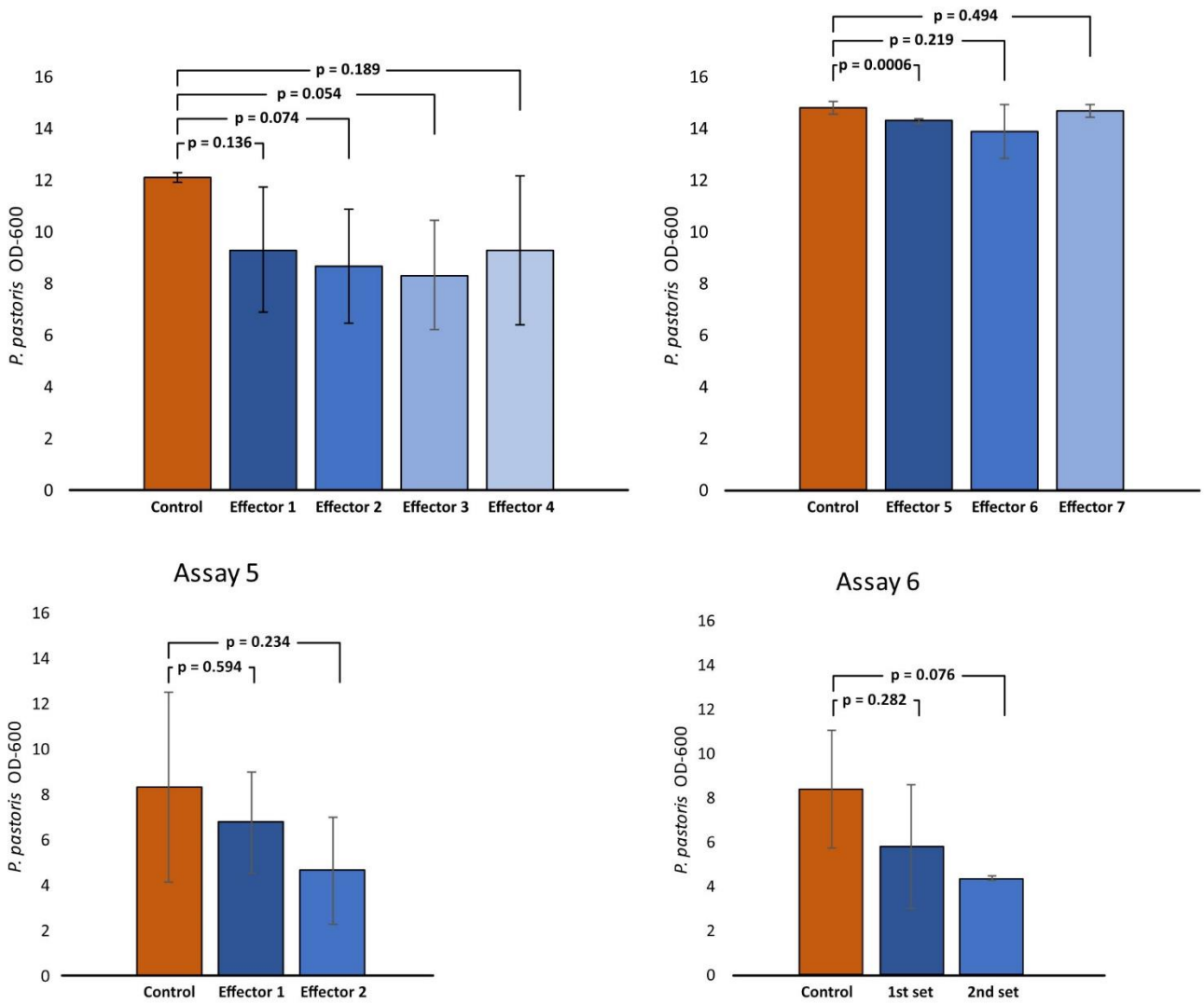

Figure S3. Results from antifungal assay showing growth of *Pichia pastoris* over 18 hours induced expression of MD-2 effector genes. Error bars indicate standard deviation.

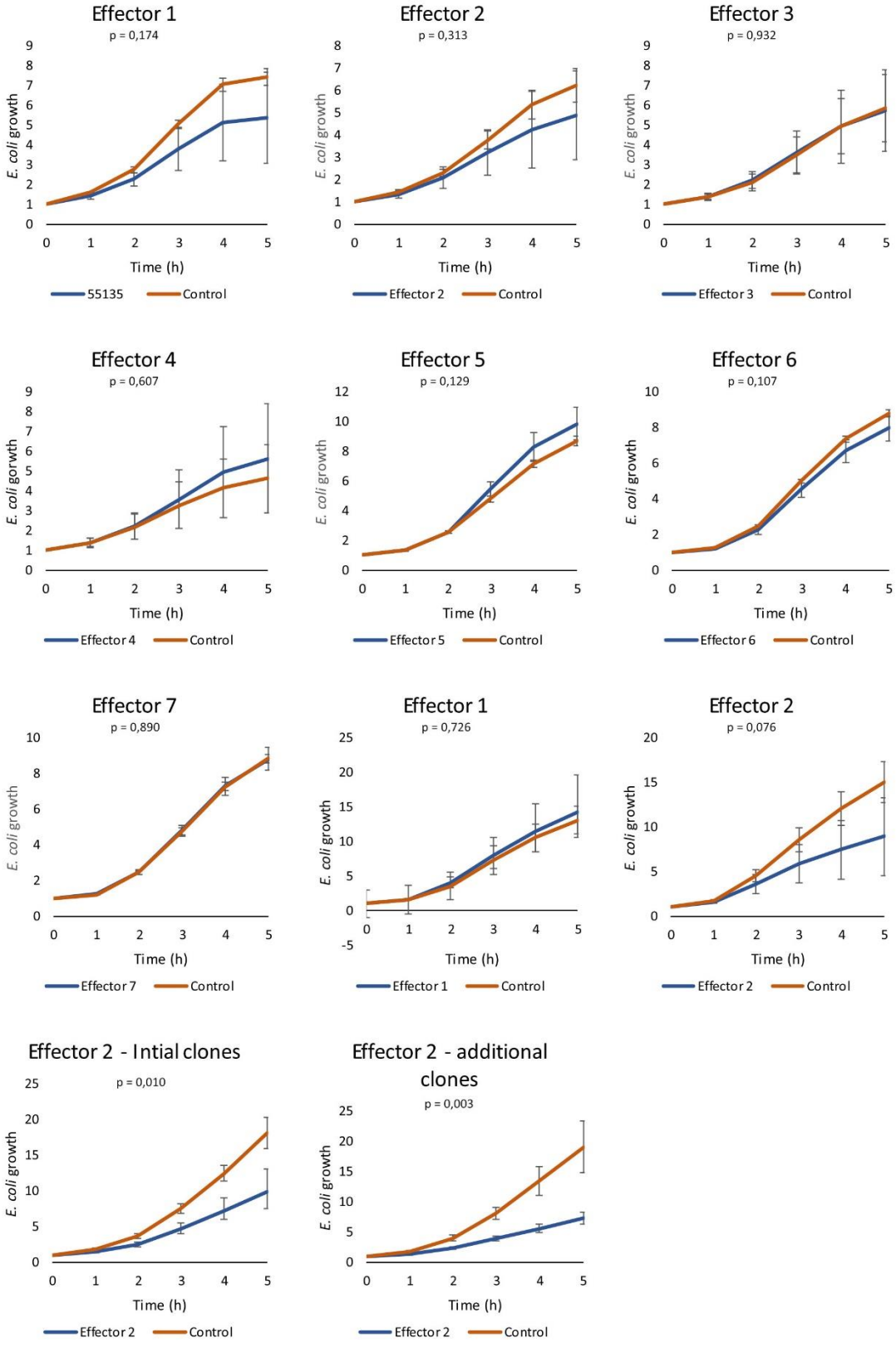

Figure S4. Antibacterial assay showing growth inhibition of *E. coli* in culture supernatant of *Pichia pastoris* transformants induced to express MD-2 effector genes.

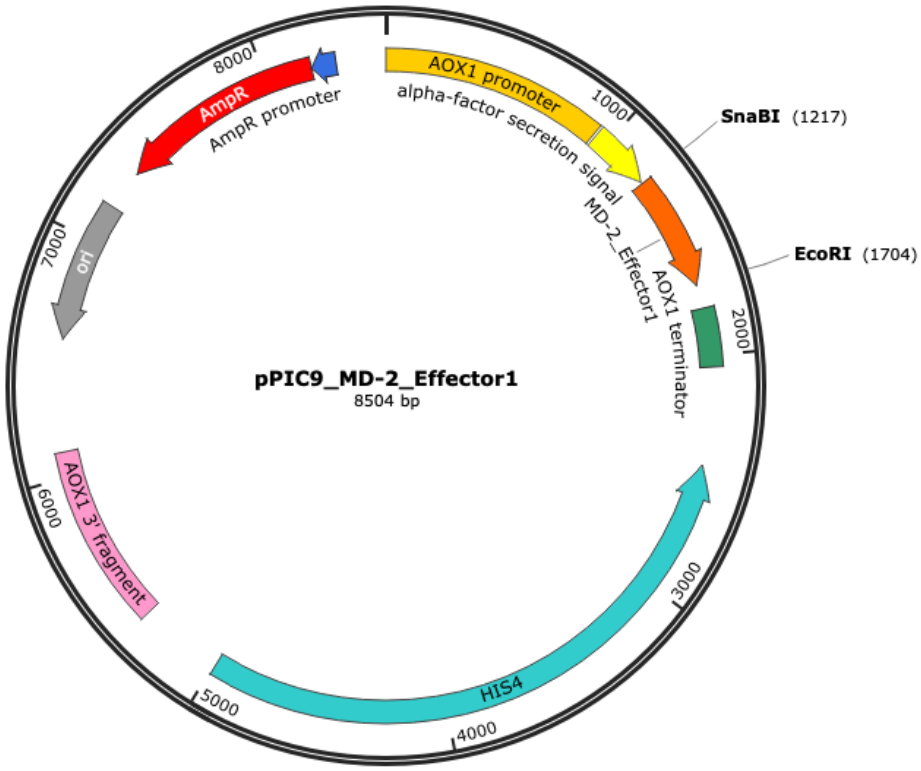

Figure S5. Transformation vector for *Pichia pastoris*. The pPIC9H vector carries an expression cassette with the AOX1 promotor, alpha-factor secretion signal, GOI and the AOX1 terminator. HIS4 and AOX1 fragments are included to promote homologous recombination at those loci respectively. The vector carries an ampicillin resistance gene as a selection marker.

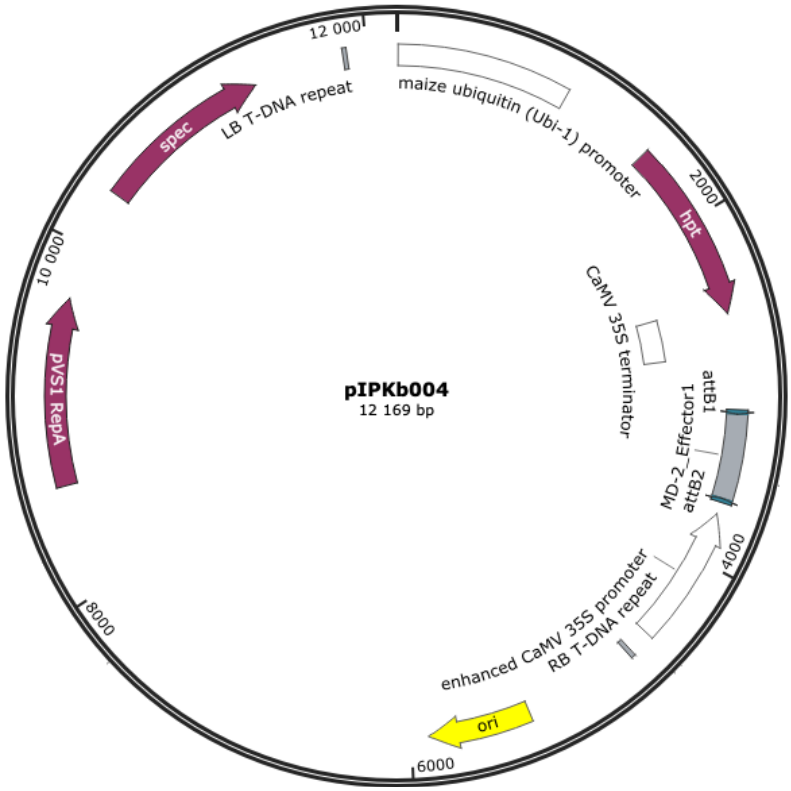

Figure S6. Binary transformation vector for *Nicotiana benthamiana*. T-DNA includes the expression cassette consisting of the enhanced CaMV 35S promoter, GOI and CaMV35S terminator. Spectinomycin and hygromycin resistence genes are included as selection markers