Exploiting the effector repertoire of *Monilinia fructicola* as a breeding strategy for disease resistance

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Abstract

***Monilinia fructicola*, *M. laxa,* and *M. fructigena* are the fungal pathogens responsible for brown rot disease in stone fruit, which can cause severe preharvest and postharvest losses. The genus *Monilinia* belongs to the family Sclerotiniaceae, which comprises a large number of plant pathogenic species with a necrotrophic lifestyle. Necrotrophic fungal pathogens kill host cells and subsequently colonize the dead tissue. Induction of programmed cell death can result from the release of metabolites or proteins with phytotoxic activity into the host plants. Such molecules are referred to as effectors. It has been shown that the induction of cell death in several pathosystems is the result of the response of the host to effectors released by nectrotrophs. The identification of effectors can play an important role in breeding for host resistance as it allows one to screen germplasm for susceptible and resistant genotypes, independent of pathogen infection tests. The objective of the present study was to utilize the genome sequence of *M. fructicola* to identify effector proteins that induce cell death in host plants. The genome was sequenced with PacBio technology and was screened for the presence of genes that encode secreted proteins and more specifically for effector proteins. A set of 134 putative effectors was identified that are presently the subject of functional studies. Several candidate effector genes were cloned into *Agrobacterium tumefaciens* for transient expression in *Nicotiana benthamiana* plants. Results indicated that some of the candidates triggered cell death. The identification and increased knowledge about effectors in pathogen virulence can in the future be exploited in effector-based selection of (partially) resistant germplasm. Our research reflects an effort to develop alternative approaches to control brown rot disease and opens a new perspective in breeding for host resistance.**

Keywords: genome, annotation, necrosis, genes

INTRODUCTION

Monilinia spp. are the causal agents of brown rot disease in stone fruit, which is recognized as the disease that causes the highest economic losses in stone fruit. *Monilinia* spp. can infect multiple plant structures resulting in blossom and/or twig blight, twig cankers, and brown rot on immature and mature fruits. *M. fructicola* belongs to the family Sclerotiniaceae and is a necrotrophic fungus (Oliveira Lino et al., 2016). Such fungi obtain nutrients from dead host cells (Dodds and Rathjen, 2010) and their infection strategies are generally believed to be less complex than those of obligate-biotrophic fungi (Horbach et al., 2011) that utilize a more complex approach to obtain nutrients and keep the host plant alive (Lo Presti et al., 2015). However, more intensive studies have revealed that the infection strategies of necrotrophic fungi are probably more complex than initially proposed (Lyu et al., 2016). Similar to (hemi) biotrophic fungi, necrotrophic fungi release effectors to either induce or suppress cell death in host plants (Lo Presti et al., 2015; Lyu et al., 2016).

The main function of effectors produced by necrotrophic fungi is to induce host cell death (Lo Presti et al., 2015). Effectors can be classified based on their structure. One group of fungal effectors consists of proteins (Lo Presti et al., 2015). Biotrophic fungi release protein effectors to suppress the innate immune system of host plants. Plants may recognize those effectors by resistance (R) proteins, leading to a programmed cell death response in the plants that prevents further spread of biotrophic fungi (Koeck et al., 2011; Lyu et al., 2016). This

defense response is often controlled by a gene-for-gene interaction (Figure 1A). Necrotrophic fungi take advantage of the presence of effector protein receptors according to the so-called inverse gene-for-gene model (reviewed in Lo Presti et al., 2015). Necrotrophic fungi release effectors that are recognized by receptor proteins, also leading to cell death. In contrast to biotrophs, however, necrotrophic fungi benefit from cell death since they are able to obtain nutrients from the dead cells (Figure 1B). In this model, plant receptors for pathogen effectors facilitate a compatible interaction between the host and the necrotrophic fungi. Therefore, in this context, these receptors can be considered to be host susceptibility proteins.

A

	Biotrophic pathogens **(Gene-for-gene model)**	
	Plant receptor present	Plant receptor absent
Pathogen effector present	Recognition→Cell Death Disease Resistance	No recognition Disease Susceptibility
Pathogen effector absent	No recognition Disease Susceptibility	No recognition Disease Susceptibility

B

	Necrotrophic pathogens **(Inverse gene-for-gene model)**	
	Plant receptor present	Plant receptor absent
Pathogen effector present	Recognition→Cell Death Disease Susceptibility	No recognition Disease Resistance
Pathogen effector absent	No recognition Disease Resistance	No recognition Disease Resistance

Figure 1. (A) Gene-for-gene model proposed for biotrophic pathogens: when a plant recognizes the pathogen effector, it triggers cell death with disease resistance as the outcome. (B) Inverse gene-for-gene model for nectrotophs: when a plant recognizes the pathogen effector, it triggers cell death with susceptibility as the outcome (adapted from Lo Presti et al., 2015).

Identification and increased knowledge on protein effectors will contribute to a better understanding of the interaction between necrotrophic fungi and host plants. Currently, bioinformatic tools, with sequence and expression data as input, are routinely used to identify potential effector proteins. The objective of the present study was to identify effector proteins present in *M. fructicola* and express them by transient expression in *Nicotiana benthamiana* plants.

MATERIAL AND METHODS

Genome assembly and gene selection

The *M. fructicola* genome was assembled with HGAP (Chin et al., 2013) and CANU (Koren et al., 2017) using PacBio data with default settings. The resulting assemblies were combined with quickmerge (Chakraborty et al., 2016), and erroneously merged contigs were manually corrected. Completeness of the assembly was assessed by the BUSCO tool (Simão, et al., 2015). Genome annotation was performed using the FUNGAP pipeline (Min et al., 2017), which included annotation by MAKER (Cantarel et al., 2008), AUGUSTUS (Stanke et al., 2006) and BRAKER (Hoff et al., 2016). Gene prediction was supported with RNA-Seq libraries isolated from infected plant tissues and in vitro tissues. Predicted proteins were manually curated and functionally annotated using the funannotate pipeline (Love et al., 2019).

Genes encoding secreted proteins were identified in the *M. fructicola* genome using several prediction tools. Signal-P v4.1 (Petersen et al., 2011) was initially used to screen for a signal peptide, followed by TMHMM v.2.0 (Krogh et al., 2001) to identify putative transmembrane domains. Proteins that lacked a signal peptide, or that had a transmembrane domain (a single transmembrane domain in the N-terminal 60 residues was allowed) were discarded. TargetP was used to predict protein localization (Emanuelsson et al., 2007). Effectors were predicted using the EffectorP tool (Sperschneider et al., 2016). From this group of genes, ten were selected that have >50 reads in the transcriptome data and a size ranging from 10 to 40 kDa, and the homolog of phytotoxic effector BcNEP2 from *B. cinerea* (Schouten

et al., 2008).

M. fructicola RNA isolation

M. fructicola isolate CPMC6 was kindly provided by Dr. Usall (IRTA, Spain). Conidia from *M. fructicola* grown in MEA plates were obtained by filtering the suspension (in water) and their concentration was estimated using a hemocytometer. 10^6 freshly harvested conidia were inoculated in 50 mL liquid 24 g L^{-1} potato dextrose medium. The *M. fructicola* suspension was incubated in an orbital shaker at 20°C and 120 rpm for 24 h. Mycelium was filtered and freeze-dried. RNA was isolated from freeze-dried mycelium using the RNeasy plant mini kit (Qiagen) according to manufacturer's instructions. The RNA concentration and quality were determined using NanoDrop 1000 spectrophotometer (Thermo Scientific). cDNA was synthesized on 1 µg of RNA template using M-MLV RT (Promega).

Primer design

Primers were designed to cover the coding sequence (CDS) of the mature candidate effector, but excluding the N-terminal signal peptide (Table 1). The length of the signal peptide was predicted for each individual protein by SignalP 4.1 using default settings. A primer was designed containing a six-nucleotides extension (CACACA) upstream of the restriction enzyme recognition site to ensure efficient cleavage – six-nucleotide recognition sequence of a compatible restriction enzyme – start or end sequence of candidate effector – 3'. The amount of nucleotides of the candidate gene was chosen to obtain a melting temperature of approximately 68°C.

Table 1. Primer design for amplification of the CDS of *Monilinia fructicola* genes.

	Forward primer	Reverse primer
Mf1	CACACA**CTCGAG**GATGGAAACTTTCCTGCTCCCG	CACACA**CTGCAG**TCAAACTACCAAGCATAGTCCTGAAATCG
Mf3	CACACA**CTCGAG**TCTCCTCATAGTCTACCAGTTGAAGCTC	CACACA**CTGCAG**TTAGTAGCCACAAGCCTTCTGTTCTC
Mf7	CACACA**GTCGAC**CTCCCAACCAATGGCGGCAC	CACACA**CTGCAG**TTACAAAATGTTAAGAGGCAAAGAGAG
Mf8	CACACA**GTCGAC**GCTCCCACTTCCTCCCCCGC	CACACA**CTGCAG**CTAAGGGGTCTTTGCCTTCCAAG
Mf9	CACACA**CTCGAG**CGTCCCGCTATCCCCG	CACACA**CTGCAG**CTATGCAGGTGGTAATGGGCAAG
Mf11	CACACA**CTCGAG**ACCCGCGACCTCGCCACCTC	CACACA**ATGCAT**CTACTTGCAGTCACTGCGACCG
MfNEP2	CACACA**GTCGAC**GCGCCATCTCAAATCGAGTCTC	CACACA**ATGCAT**CTAGAAAGTAGCCTTGCCAAGGTTG

cDNA amplification of candidate effector genes

Amplification of candidate effector cDNA was performed in a total volume of 50 µL containing 0.2 µM of both forward and reverse primer, 0.2 mM of each dNTP, 1x PFU buffer (Promega), 1.5 U PFU polymerase (Promega), and 2 µL of template. Template consisted of 40 ng cDNA from *M. fructicola* mycelium. The amplification was performed in a thermal cycler using a PCR program of 95°C for 2 min, followed by ten cycles of 95°C for 30s, 58°C for 30 s and 72°C for 2 min, followed by 25 cycles with an annealing temperature of 60°C instead of 58°C. The program ended with a final step at 72°C for 5 min. Products of candidate effector gene amplification and colony PCR were run in 1-3% agarose gels. The product size and concentration was determined using 500 ng of either 1 kb DNA ladder (Promega) or quick load purple low molecular weight DNA ladder (Biorad). The products were visualized and photographed by ChemiDoc MP Imaging System (Biorad).

Digestion of PCR products and plasmid

PCR products and plasmids were digested using Fast digest enzymes (Thermo Scientific), using either *Xho*I, *Pst*I, *Sal*I, or *Mph*1103I (*Nsi*I) in 1x Fast digest® buffer (Thermo Scientific) at 37°C. The incubation and inactivation time for each enzyme was based on the manufacturer's protocol. PCR products, digested constructs, and digested plasmids were cleaned using the DNA purification kit from Macherey-Nagel.

Ligation of gene constructs and *E. coli* transformation

Digested and cleaned plasmids and PCR products were ligated using T4 DNA Ligase

(Promega) using a 5:1 molar ratio of insert PCR product:vector. The reaction was incubated at 14°C overnight. The ligated plasmid was added to 50 µL of ultra-competent DH5α *Escherichia coli* cells and mixed gently. After incubation on ice for 20 min, a heat-shock treatment was performed and SOC medium was added. The transformed cells were incubated while shaking at 37°C for 1 h and plated on LB medium with 50 µg mL^{-1} kanamycin overnight.

Colony PCR and plasmid isolation

The insert of transformed colonies grown on selection plates were checked using colony PCR. Half of the colony was suspended in water and used in a 25 µL PCR reaction using GoTaq polymerase (Promega). The forward primer (5' CTCCACTGACGTAAGGGATGACGCAC) annealed to the vector while the reverse primer was specific for the effector gene amplicon (Table 2). The PCR product was checked using agarose gel electrophoresis. Colonies containing the correct PCR amplicon were grown in LB medium containing 50 µg mL^{-1} kanamycin overnight. Plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's protocol and the isolated plasmids were sequenced with the Mix2Seq kit (Eurofins Genomics).

Table 2. Overview of selected candidate effector genes of *Monilinia fructicola*.

Gene names	*M. fructicola* gene code	Protein size	Number of cysteines	In planta expression	In vitro expression	Predicted domain
Mf1	MFRU_002 g05260	221	10	198	299	CFEM
Mf2	MFRU_002 g02190	94	10	4060	31	
Mf3	MFRU_012 g01440	245	4	682	12129	Tuberculosis necrotizing toxin
Mf4	MFRU_012 g01930	191	4	1592	326	
Mf6	MFRU_028 g01250	149	5	97	1318	Cerato-platanin
Mf7	MFRU_030 g00190	126	6	44033	684849	
Mf8	MFRU_030 g00580	160	6	3652	556	
Mf9	MFRU_034 g00500	82	8	5431	1966	
Mf10	MFRU_034 g00870	95	10	64	969	CFEM
Mf11	MFRU_048 g00370	147	9	120	63	
Mf13	MFRU_072 g00020	242	5	280	709	Necrosis inducing protein

***Agrobacterium* transformation**

Fifty nanograms of plasmid was added to 50 µL of electro-competent *Agrobacterium tumefaciens* cells (strain GV3101). The electroporation was performed in the Gene Pulser Xcell (Biorad) using the settings 2400 V, Cap 25, Res 200, 2 mm. After 5 min on ice, 900 µL of SOC medium was added to the transformed cells. The cells were incubated at 28°C for 90 min without shaking, and spread on a LB plate containing kanamycin, gentamicin and rifampicin. Transformed *Agrobacterium* colonies were obtained after three days of incubation at 28°C.

Transient gene expression in *N. benthamiana*

A single transformed *Agrobacterium* colony was grown in 15 mL YEB medium supplemented with 20 µM acetosyringone, kanamycin, gentamicin and rifampicin at 28°C. The culture was centrifuged and re-suspended in MMAi buffer at OD_{600}=0.8. After two hours of incubation, the cells were infiltrated using a needleless 1 mL syringe (Omnifix®-F, Braun) in leaves of 4- to 6-week-old *N. benthamiana* plants. Pictures of the response in *N. benthamiana* leaves were taken at five to six days post-infiltration.

RESULTS

The genome of *M. fructicola* isolate CPMC6 was assembled into 99 contigs with a total length of 42.95 Mb. Gene prediction tools followed by manual curation and functional annotation resulted in a set of 10,086 high-confidence proteins, of which 134 are putative effector proteins (Figure 2). From this list, ten candidate effector genes were selected based on high levels of gene expression in vitro and *in planta*, the presence of multiple cysteine

residues, low protein size, and predicted cell death-inducing domains.

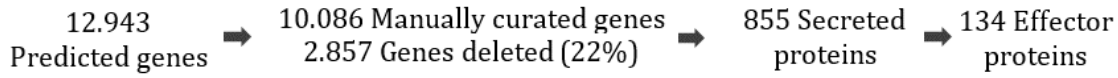

Figure 2. Process to obtain the genes that encode putative effector proteins in *Monilinia fructicola* genome.

Homologs of established cell death-inducing proteins were also identified in the *M. fructicola* genome, and included in our analysis. Necrosis and ethylene-inducing peptides (NEPs) are well characterized proteins in microbes that induce cell death in dicotyledonous plants. *Botrytis cinerea,* a fungus closely related to *M. fructicola*, contains two NEP proteins, BcNEP1 and BcNEP2 (Arenas et al., 2010). MFRU_014 g02060.1 (MfNEP1) and MFRU_072 g00020.1 (MfNEP2) exhibited 73% and 88% amino acid identify to BcNEP1 and BcNEP2, respectively.

Primers were designed for full CDS amplification of the *M. fructicola* candidate effector genes, and *MfNEP1* and *MfNEP2*. The aim was to amplify the CDS encoding the mature effector protein (lacking the signal peptide) from fungal cDNA instead of genomic DNA, to avoid problems with inefficient splicing of fungal splice junctions in the *N. benthamiana* expression system. Amplicons were obtained for all genes except for *Mf2*, *Mf4*, and *Mf10*. After cloning the constructs into a binary vector containing the CaMV 35S promoter and a plant signal peptide sequence suitable for transient protein production in the plant apoplast, the presence of the insert was checked in *E. coli* cells using colony PCR. *Mf1*, *Mf3*, *Mf7*, *Mf8*, *Mf9*, *Mf11* and *MfNEP2* were successfully cloned and sequencing of the plasmids confirmed the correct insertion of the construct into the vector, in-frame with the plant signal peptide. Despite multiple attempts, correct clones for *Mf6* and *MfNEP1* could not be obtained. Transient expression of Mf1, Mf8, and MfNEP2 by agroinfiltration resulted in cell death in infiltrated areas (Figure 3).

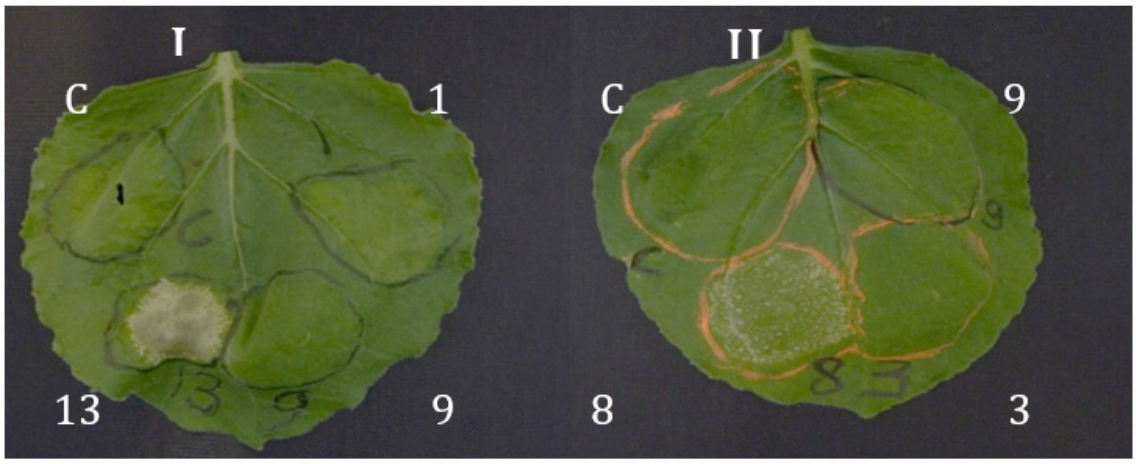

Figure 3. Transient expression of *M. fructicola* candidate effector genes in *N. benthamiana* using a binary vector for expression in plants. (I) Clockwise from top left, empty-vector (c), Mf1 (1), Mf9 (9) and MfNEP2 (13); (II) clockwise from top left, empty-vector (c), Mf9 (9), Mf3 (3) and Mf8 (8). Pictures were taken six days post infiltration.

DISCUSSION

Purified fungal effectors are valuable in breeding programs, since effectors can be used for rapid screening of germplasm to detect susceptible plant accessions in a more efficient

manner, independent of pathogen infection tests (Vleeshouwers and Oliver, 2014). Plant accessions that are less sensitive to effector proteins are likely to also be less susceptible to the infection by necrotrophic fungi. Only limited molecular studies have been published thus far on the stone fruit pathogen *M. fructicola*, and cell death-inducing proteins have not been reported. Therefore, this study aimed to identify host-specific, cell death-inducing effectors of *M. fructicola*.

Cell death-inducing capacity was observed upon transient expression of *M. fructicola* candidate effectors Mf1, Mf8 and MfNEP2 in *N. benthamiana*. MfNEP2 is a homolog of BcNEP2, which has been characterized as a cell death-inducing protein in *B. cinerea* (Schouten et al., 2008). MfNEP2, similar to BcNEP2, contains a GHRHDWE motif, which is a hallmark of NEP1-like proteins, NLPs (Arenas et al., 2010). This heptapeptide motif is not found in other proteins, suggesting that MfNEP2 could also be classified as a NLP. NLPs are known for their cell death-inducing capacity in a broad range of dicotyledonous plant species (Schouten et al., 2008). The GHRHDWE motif was also found in the *M. fructicola* protein referred to as MfNEP1. MfNEP1 is a homolog of BcNEP1, which was observed to be a stronger inducer of cell death than BcNEP2 (Schouten et al., 2008). Unfortunately, cloning efforts for MfNEP1 were not successful at this point. BcNEP1 and BcNEP2 are known to be expressed at different time points during *B. cinerea* disease development (Arenas et al., 2010), and the same is true for SsNEP1 and SsNEP2 in *S. sclerotiorum* (Dallal Bashi et al., 2010). MfNEP1 was found to be highly expressed only during primary lesion formation, while MfNEP2 exhibited high expression during primary lesion formation and further increased during lesion expansion of *M. fructicola* in nectarine leaves (Vilanova et al., unpublished). The cell death-inducing capacity of MfNEP2, together with the enhanced expression during infection, suggests that this gene may contribute to the induction of cell death during the interaction of *M. fructicola* with its hosts.

In addtion to MfNEP2, this study also identified Mf8 as a strong inducer of cell death in *N. benthamiana* leaves. Mf8 has no known domains, but was included in the research because of the presence of 6 cysteine residues, the small protein size (160 amino acids), and the relatively high gene expression during fruit infection. The expression of Mf8 in nectarine leaves was highest at 48 h after inoculation, which corresponds with primary lesion formation of *M. fructicola* on nectarine leaves (Vilanova et al., unpublished). The upregulation of Mf8 in the early phase of the infection process, together with the cell death-inducing capacity, suggests that Mf8 plays a role in the *M. fructicola*-host interaction.

Necrotic symptoms were observed as a result of the transient expression of Mf1 in *N. benthamiana*. Although the symptoms were typically delayed and less severe than for MfNEP2 and Mf8, necrotic symptoms for Mf1 were observed in multiple experiments. Mf1 contains a PFAM domain called CFEM, characterized by its eight cysteine residues with a specific spacing (Kulkarni et al., 2003). The CFEM domain is a unique fungal domain, often present in proteins playing a role in fungal pathogenesis (Kulkarni et al., 2003). Fungal proteins containing a CFEM domain are often anchored to the cell surface through a glycosylphosphatidylinositol (GPI)-anchor and/or by glycosylation (Kou et al., 2017; Zhu et al., 2017). These CFEM domains are proposed to function as cell surface receptors or adhesion molecules in host-pathogen interactions (Kou et al., 2017). Zhu et al. (2017) identified a CFEM protein (BcCFEM1) in *B. cinerea*, with GPI-modification sites. A *ΔBcCFEM1* deletion mutant showed reduced lesion size compared to the WT *B. cinerea* strain on bean leaves, indicating that BcCFEM1 is a virulence factor of *B. cinerea*. In contrast to most reported CFEM proteins, the cell death-inducing candidate effector Mf1 lacks GPI-modification sites and transmembrane domains. Only limited studies are available on fungal proteins with a CFEM domain that are not attached to the cell membrane and thus the role of Mf1 in virulence of *M. fructicola* is still unclear.

In summary, three proteins of the stone fruit pathogen *M. fructicola* have cell death-inducing capacity in *N. benthamiana.* The activity of these proteins in tissues of stone fruit trees remains to be tested. These results present an interesting novel approach to investigate the cell death-inducing effectors of *M. fructicola* and to utilize them to screen for (partially) resistant germplasm.

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