Identification and characterization of *Dothistroma septosporum* effectors

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Yanan Guo

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Abstract

Dothistroma septosporum is the main causal agent of Dothistroma needle blight of pines. However little is known about mechanisms of pine resistance against *D. septosporum*, or whether there is any classical gene-for-gene resistance involved. The molecular basis of how fungal effector proteins can trigger plant host resistance in a gene-for-gene manner was determined partly by work with the model fungus *Cladosporium fulvum* and its tomato host. Comparative genome analysis of *C. fulvum* and *D. septosporum* genomes identified nine putative effector genes (*DsAvr4*, *DsEcp2-1*, *DsEcp2-2*, *DsEcp2-3*, *DsEcp4*, *DsEcp5*, *DsEcp6*, *DsEcp13* and *DsEcp14*) in *D. septosporum* that are homologous to well-characterized *C. fulvum* effector genes. Other effector candidates were identified as small cysteine rich proteins that are highly expressed *in planta*, including *DsHdp1* which is a hydrophobin gene and *Ds69335* which belongs to the sperm-coating protein-like extracellular protein SCP/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) superfamily.

Transcriptome analysis showed that, except for *DsEcp2-1* and *DsEcp6*, the *in planta* expression of *D. septosporum* effectors was low. Targeted gene replacement of *DsAvr4*, *DsEcp2-1*, *DsEcp6* and *DsHdp1* caused no observed changes in fungal physiology *in vitro* compared to wild type (WT) and also showed that *DsAvr4*, *DsEcp6* and *DsHdp1* are not virulence factors when infecting *Pinus radiata*. However deletion of *DsEcp2-1* caused larger lesions compared to WT, suggesting that DsEcp2-1 may act to suppress a host target which is involved in necrosis induction during the biotrophic infection stage.

A domain swap experiment in this study showed that swapping the region between cysteine residues C6 (Cys102) to C7 (Cys114), which contains the chitin binding domain, caused loss of resistance (R) protein Cf-4 recognition of DsAvr4 (with CbAvr4) and gain of Cf-4 recognition of CbAv4 (with DsAvr4 or CfAvr4). Further experiments carried out in Wageningen University showed that a Pro residue located in the chitin binding domain in DsAvr4 is important for Cf-4 recognition, and may have a role in DsAvr4 stability. In this study, effector candidates DsEcp2-1 and DsEcp2-3 were able to trigger a non-host necrotic response in *N. tabacum* suggesting possible interaction with a *N. tabacum* protein. Polymorphism analysis showed that *DsEcp4* and *DsEcp5* have internal stop codons and encode pseudogenes in all the *D. septosporum* strains tested,

except for *DsEcp4* in strains from Guatemala and Columbia in which a functional gene is predicted. DsEcp4 and DsEcp5 are the only *D. septosporum* effectors tested that showed evidence of positive selection. Those results lead to the suggestion that R proteins that recognise DsEcp4 and DsEcp5 may be present in pine species. *DsEcp13* appears to be absent from ten *D. septosporum* strains, suggesting that DsEcp13 is not important for virulence and can also be deleted to avoid an R protein mediated defence response such as a hypersensitive response. Infiltration of DsAvr4, DsEcp2-1 and DsEcp6 *P. pastoris* expression culture filtrates triggered necrosis in *P. radiata* needles suggesting that R proteins that directly or indirectly recognise those effectors may also be present in *P. radiata*.

The finding that *D. septosporum* has homologues of *C. fulvum* effectors allowed the first study of molecular pathogen-host interactions in this pathosystem. Targeted gene replacement studies identified genes that may have a virulence function and resistance against these effectors may be durable in the field. The pine needle infiltration assay provides a basic screening method to identify pine genotypes that carry resistance proteins and future work in this area is expected to impact on breeding strategies in the forest industry.

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Abbreviations

Abbreviation	Meaning
Avr	avirulence protein
CC:	coiled-coil domain
ChBD	chitin-binding domain
Cys	cysteine residue
Dnase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
Ecp	extra cellular protein
eLRR	extracellular LRR
G	gram
HR	Hypersensitive response
IPTG	isopropyl-β-d-thiogalactoside
kb	kilobase pair
L	litre
LRR	leucine-rich repeat domain
M	molar
Mb	megabase
ml	milliliter
mM	millimolar
NBS	nucleotide-binding site
ng	nanogram
NLP	necrosis and ethylene-inducing like protein
NLS	nuclear localization signal
ORF	open reading frame
PAMPs	pathogen-associated molecular patterns
PCD	programed cell death
PCR	polymerase chain reaction
PEST	Pro-Glu-Ser-Thr domain
PRRs	plant PAMP-recognition receptors
PTI	PAMP triggered immunity
qPCR	real-time PCR
R protein	resistant protein
RGAs	Rprotein analogs
RLKs	receptor-like kinase proteins
RLPs	receptor like protein
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSCP	small secreted cysteine rich protein
T3SS	bacterial type III secretion system
TIR:	toll-interleukin 1 receptors
TM	transmembrane domain
UV	ultraviolet
WRKY	DNA binding domain
WT	wild type
X-Gal	5- bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	microgram
µl	microlitre
µM	micromolar