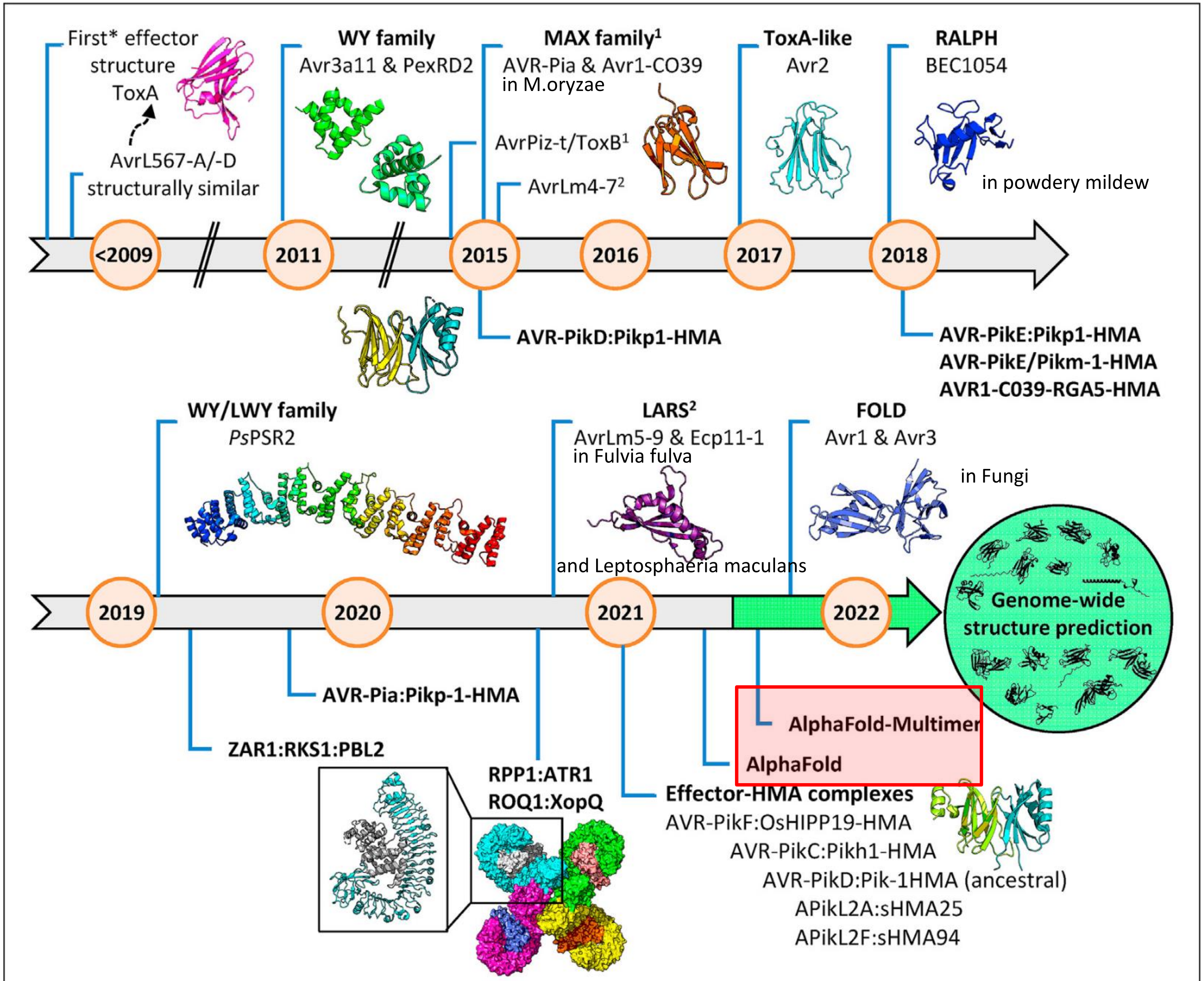
Killer proteins 4 and 6 from the fungal wheat pathogen *Zymoseptoria tritici* are toxic to fungi and structurally related to fungal effector families

Main advances in the study of fungal effector protein structures

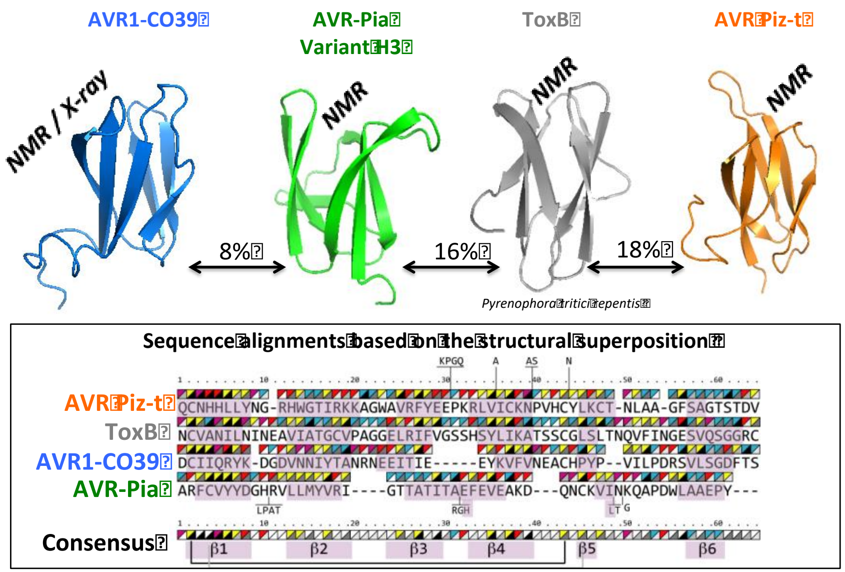


Outram et al, Current Opinion in Plant Biology, 2022

Novel effectors
from *Zymoseptoria tritici*
a fungal pathogen of wheat

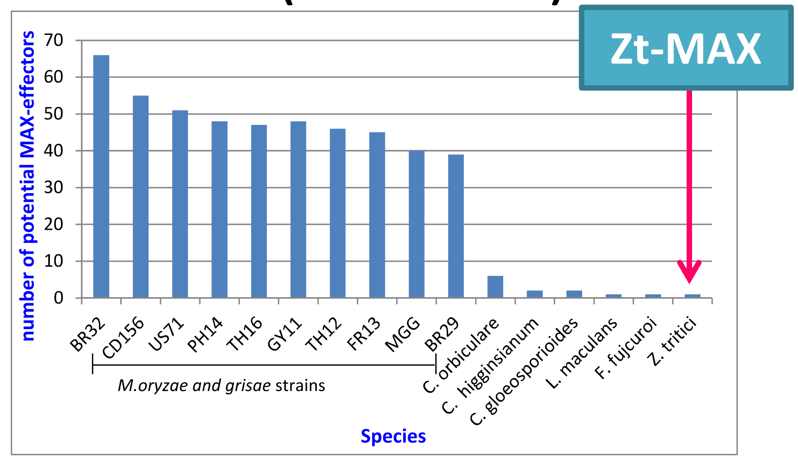
Could structural analysis
help classify these effectors ?

MAX effectors



Different primary sequences but similar structure (shared fold : MAX effectors)

Max effector (HMM search)



One candidate MAX in *Zymoseptoria*

Low sequence similarity

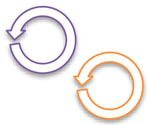
Is it really a MAX effector ?

↓

3D structure determination

de Guillen et al. PLoS Pathog. 2015. 27;11(10):e1005228.

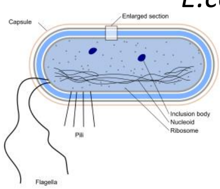
Structural biology analysis



Cloning

Transformation

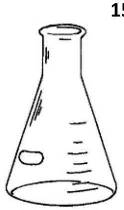
E.coli

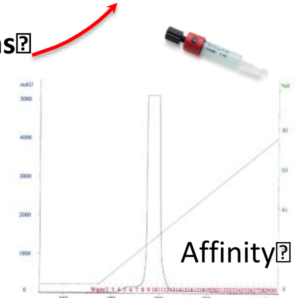


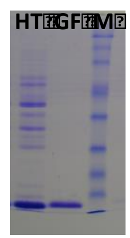
Productions

^{15}N

^{13}C

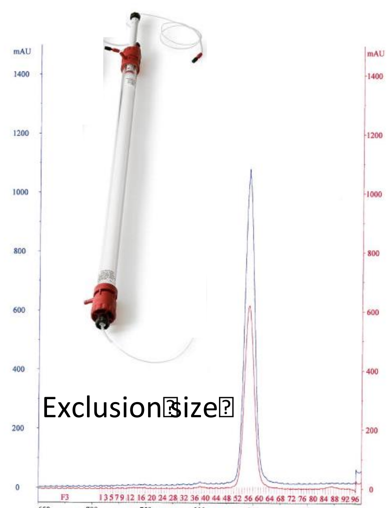






Purifications by chromatography

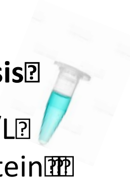
Affinity



Exclusion size

Structural analysis

20 mg/L
pure protein



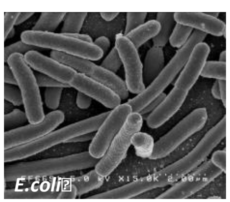
highly pure protein

X-RAY

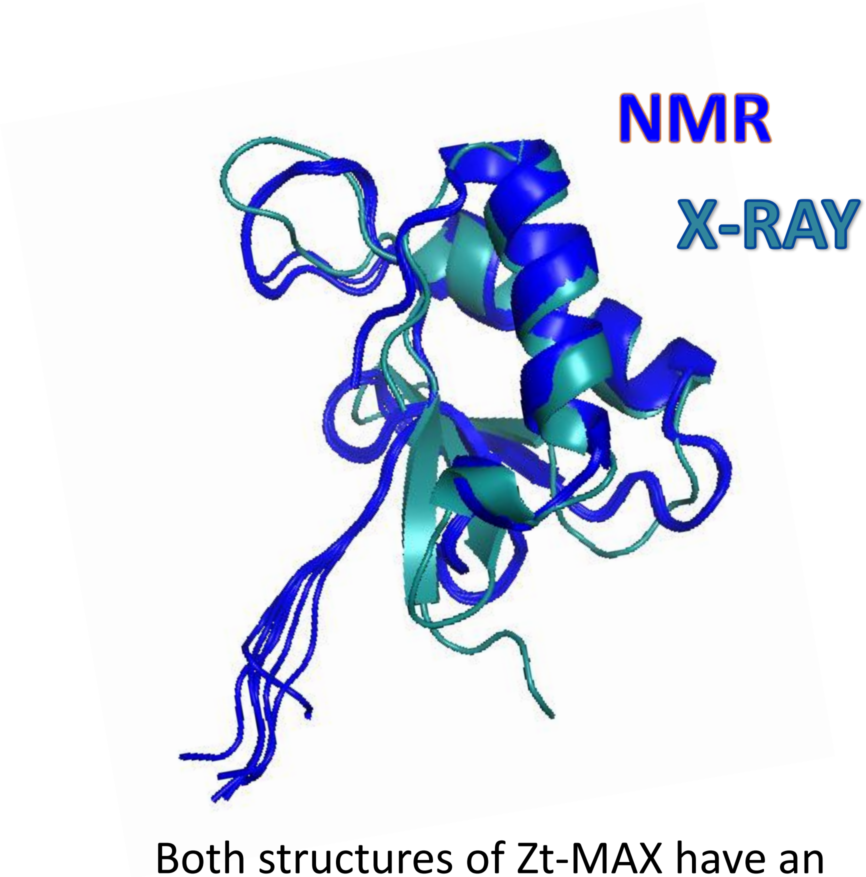
Solid structure :
Crystals
Diffraction pattern
Phase solving

NMR

Structure in solution :
protein dynamics

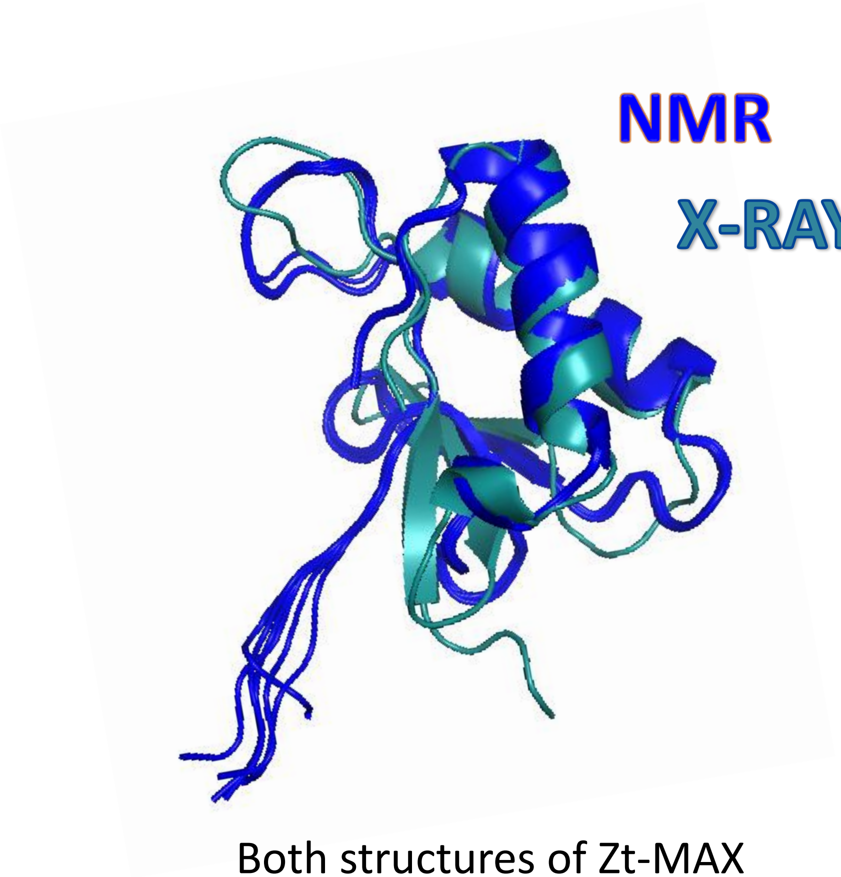


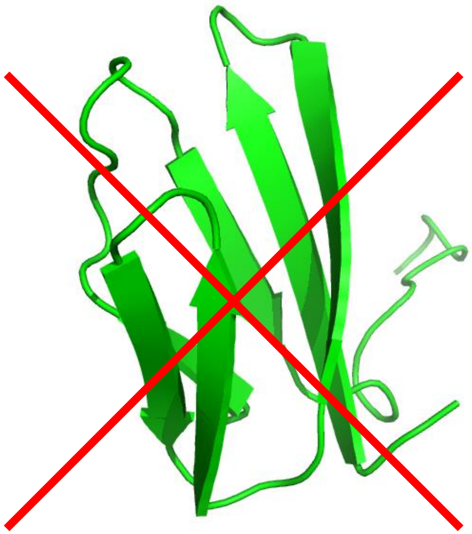
3D structure of Zt-MAX



Both structures of Zt-MAX have an
α/β structure
with tree stranded β-sheet
and two α-helices.

3D structure of Zt-MAX





Both structures of Zt-MAX have an **α/β structure** with tree stranded β-sheet and two α-helices.

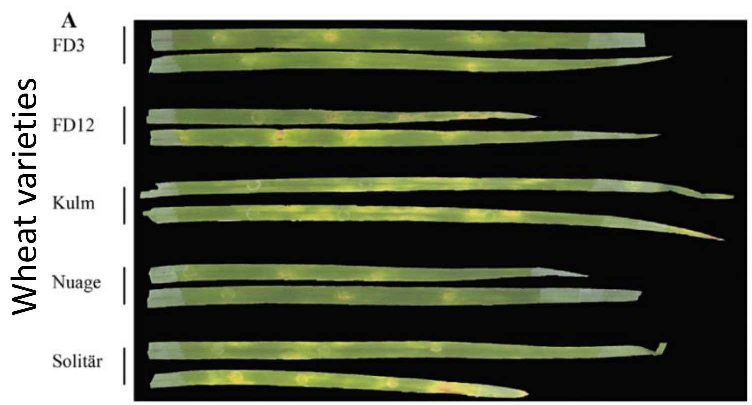
MAX effectors have a **β sandwich structure** of six antiparallel β strands.

Zt-MAX is not a MAX effector.

Zymoseptoria tritici effector protein NIP1

Zt-NIP1

Zt-NIP1 a small secreted protein inducing wheat leaf necrosis expressed during infection: **Candidate effector toxic to wheat**

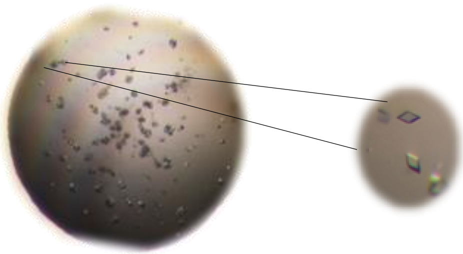


Necrosis-inducing activity of the ZtNIP1

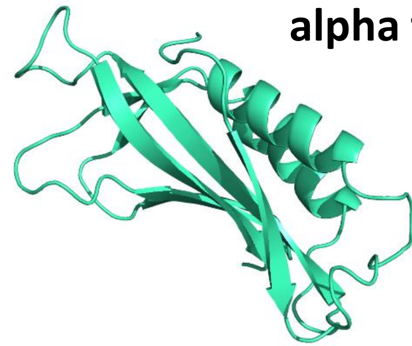
Ben M' Barek et al., 2015, Fungal Genet Biol. 79:54-62.

Zt-NIP1 3D structure

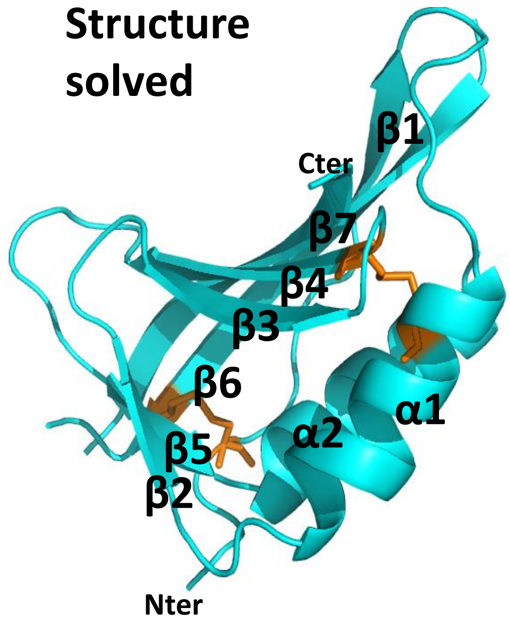
X-RAY analysis



Molecular replacement using alpha fold model



Structure solved

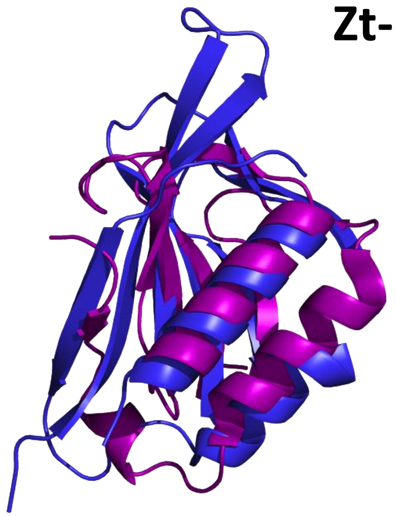


NIP has an **α/β structure**
seven β strands,
two α helices
and two disulphide bonds:

Structural homologs of Zt-NIP1 and Zt-MAX

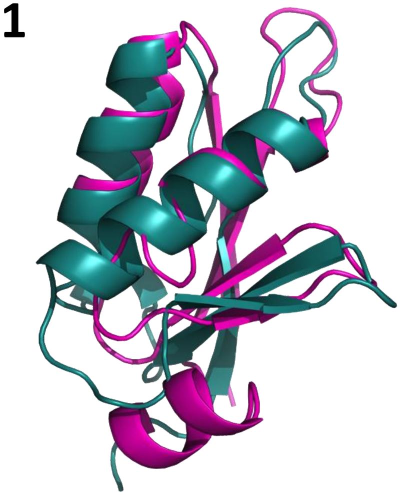
Search using DALI
PROTEIN STRUCTURE COMPARISON SERVER

Zt-KP4-1 **Zt-KP6-1**



Zt-NIP1 / UmV-KP4
PDB: 8ACX PDB:1KPT

RMSD: 2.5 Å
Z-Score: 6.7



Zt-MAX / UmV-KP6α
PDB: 6QPK PDB:1KP6

RMSD: 2.4 Å
Z-Score: 7.3

UmV-KP4 and UmV-KP6α are fungitoxic proteins of *Ustilago maydis* RNA virus (UmV)

Zt-NIP1 → Zt-KP4-1
Zt-MAX → Zt-KP6-1

What are UmV-KP4 and UmV-KP6?

KP4 and KP6 killer toxins are encoded by different strains of
dsRNA virus (UmV) infecting *Ustilago maydis*, a fungal pathogen of maize

Maize Smut Fungus
(*Ustilago maydis*)

These virus encoded proteins are toxic to fungi
- Only non-infected *U. maydis* strains are sensitive to toxins
- Infected *U. maydis* are immune to the toxin they produce :

Killer phenotype: infected strains kill non-infected strains

KP4 fungal toxin inhibits growth in *Ustilago maydis* by blocking calcium uptake

Matthew J. Gage,[1] Jeremy Bruenn,[2] Marc Fischer,[3] Dale Sanders[3] and Thomas J. Smith[1]
[1]*Donald Danforth Plant Science Center, 7425 Forsyth Boulevard, Box 1098, St Louis, MO 63105, USA.*
[2]*Department of Biological Sciences, SUNY/Buffalo, Buffalo, NY 14260, and Hauptman-Woodward Medical Research Institute, Inc., 73 High St., Buffalo, NY 14203-1196, USA.*
[3]*Biology Department, University of York, PO Box 373, York YO1 5YW, UK.*

segments present in some multisegmented dsRNA viral genomes. The inhibitory factors (killer toxins) were shown to be secreted proteins encoded by the single dsRNA segments (Hankin and Puhalla, 1971). A small proportion of *U. maydis* cells can produce killer toxins, to which they are resistant; sensitive cells are the majority in wild populations. Killer toxins have been identified in eight genera of yeast (Young, 1987), but the killer toxins of *Ustilago* are the only ones known in a filamentous fungus. The *U. maydis* killer toxins are effective against species in

ELSEVIER
bms British Mycological Society promoting fungal science
journal homepage: www.elsevier.com/locate/

Review

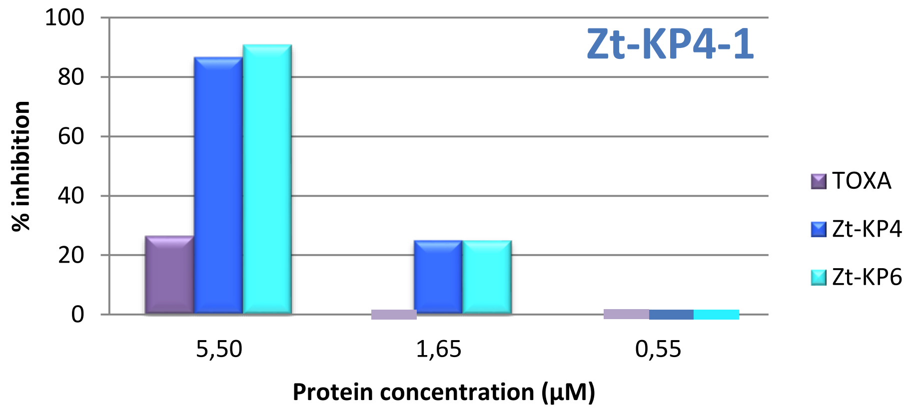
The virally encoded killer proteins from *Ustilago maydis*

Aron ALLEN[a], Emir ISLAMOVIC[b,1], Jagdeep KAUR[a], Scott GOLD[b], Dilip SHAH[a], Thomas J. SMITH[a,*]
[a]Donald Danforth Plant Science Center, 975 North Warson Road, Saint Louis, MO 63132, USA
[b]Department of Plant Pathology, University of Georgia, Athens, GA 30602, USA

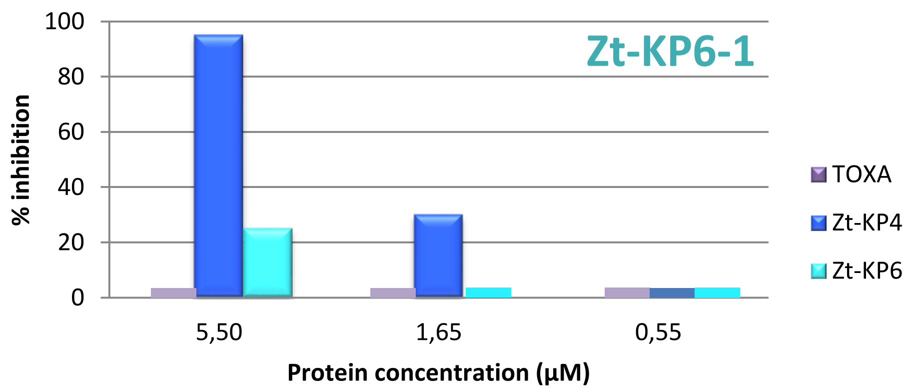
Are Zt-KP4-1 and Zt-KP6-1 toxic to fungi ?

Fungitoxicity assays

***Botrytis cinerea* growth**



***Zymoseptoria tritici* growth**



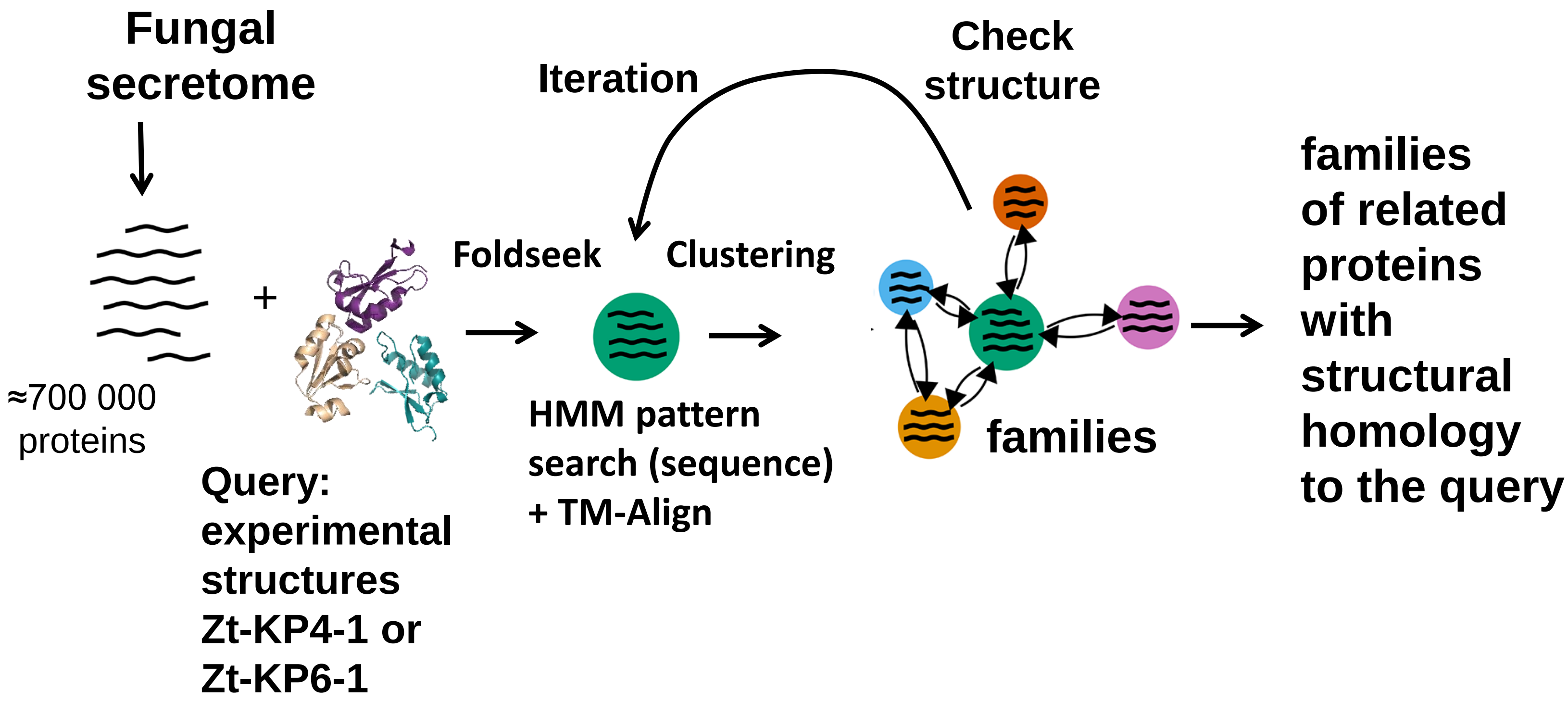
Zt-KP4-1 is fungitoxic to both *B. cinerea* and *Z. tritici*

Zt-KP6-1 is fungitoxic to *B. cinerea*
***Z. tritici* is less susceptible**
(4-fold lower sensitivity than *B. cinerea*)

No toxicity of Zt-KP4 and Zt-KP6-1 to wheat leaves

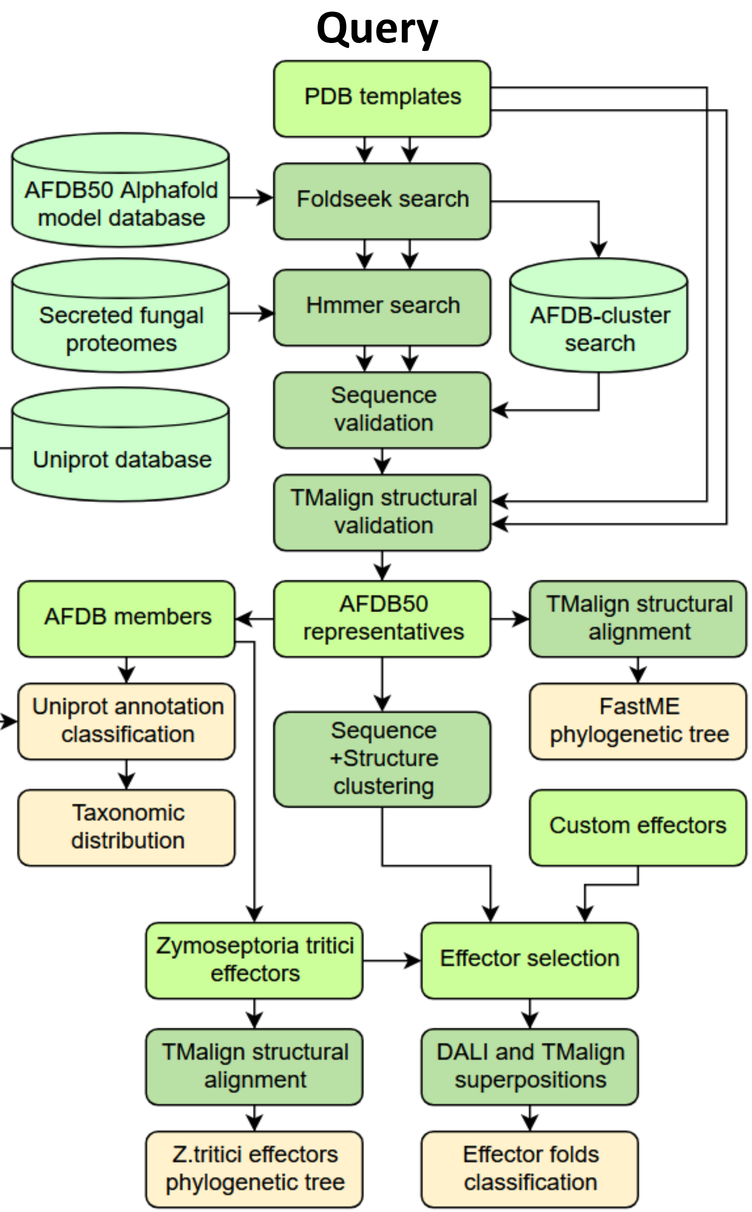
Negative control : TOXA, phytotoxic effector from *Parastagonospora nodorum*

Search for Zt-KP4 and Zt-KP6 like proteins in fungi

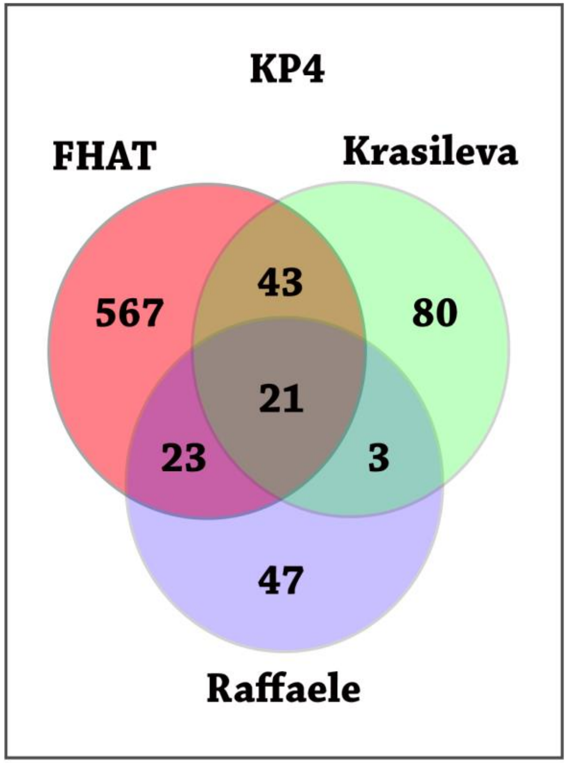


https://search.foldseek.com/search

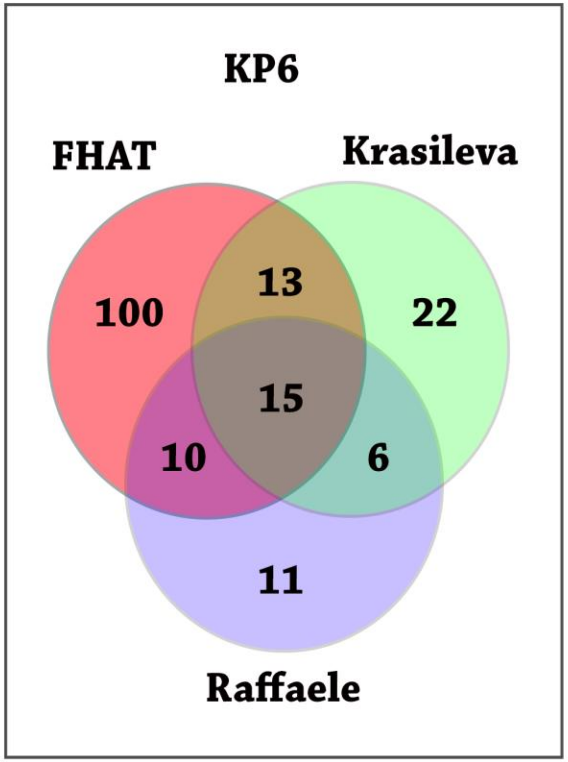
Search for Zt-KP4 and Zt-KP6 like proteins in fungi



Search for Zt-KP4 and Zt-KP6 like proteins in fungi



Our search for KP4
654 proteins
(60 families): FHAT



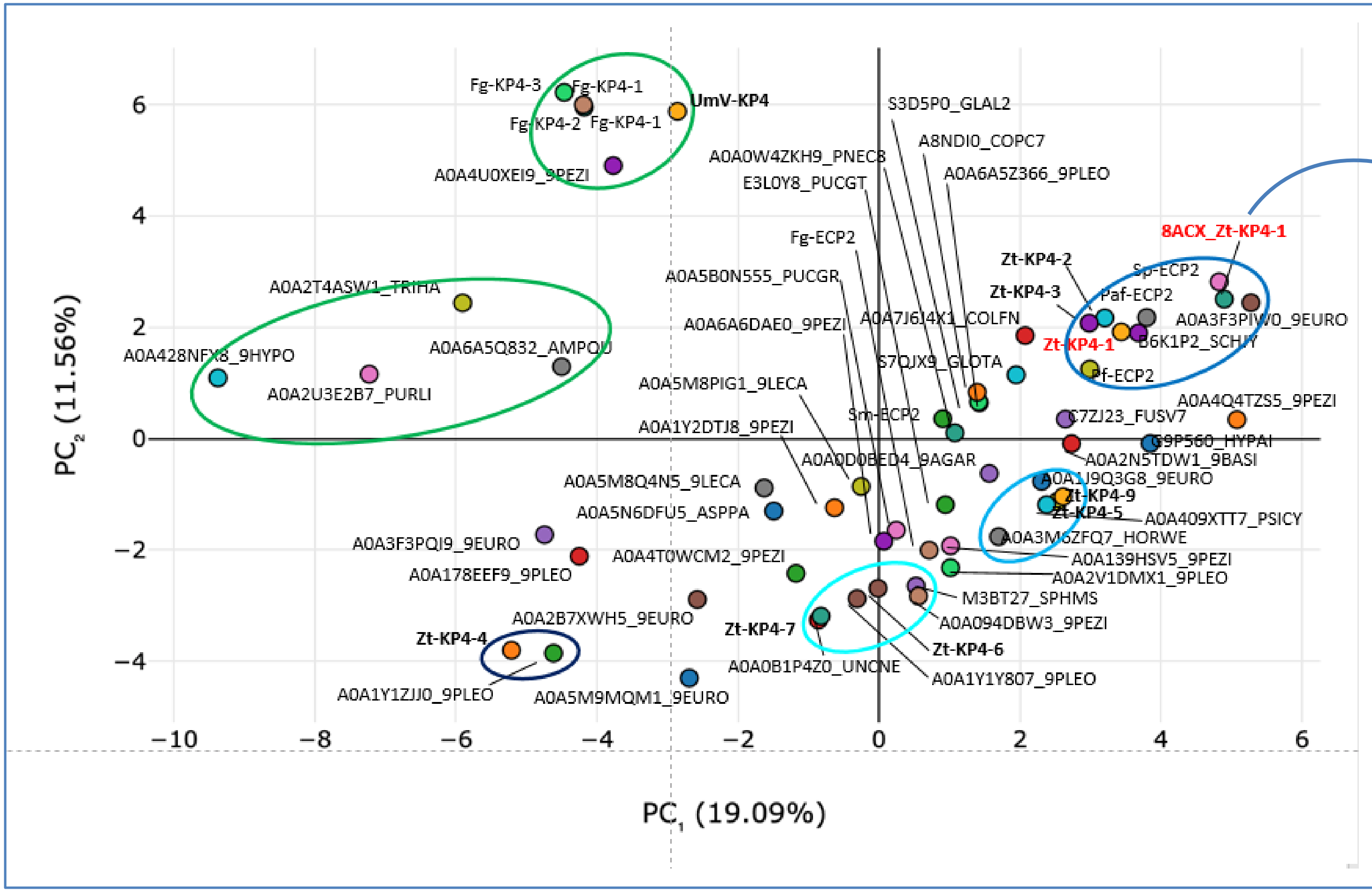
Our search for KP6
138 proteins
(30 families): FHAT

Seong K, Krasileva KV. Nat Microbiol. 2023, 8(1):174-187.

Derbyshire MC, Raffaele S. Nat Commun. 2023, 14(1):5244.

Classification of fungal Zt-KP4 like proteins

PCA analysis using the structural distances between KP4 like proteins (Z-score DALI)



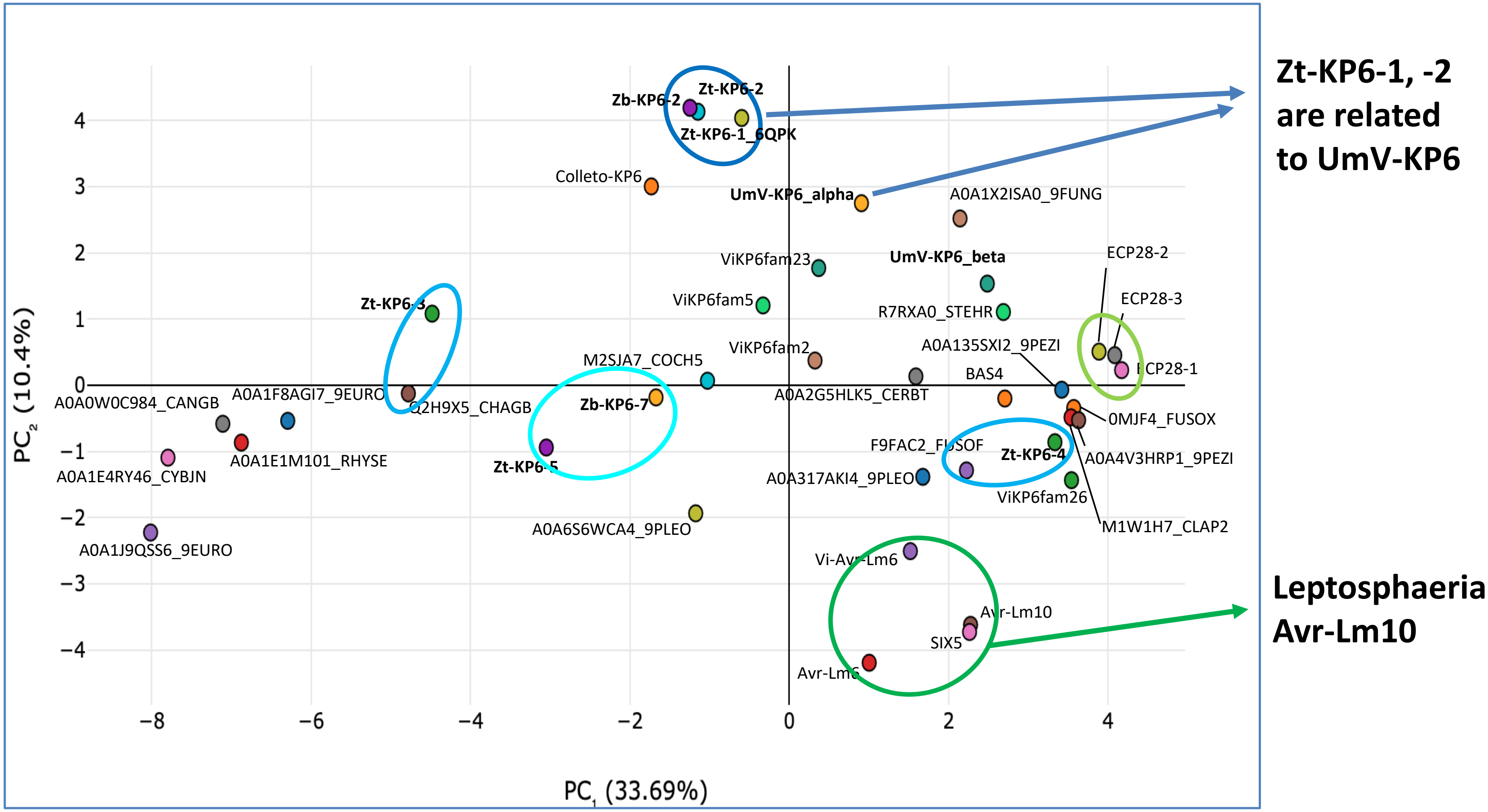
Zt-KP4-1, -2, -3 are related to ECP2 effector family

9 Zt-KP4 like proteins in *Zymoseptoria*

Zt-KP4-1, -2, -3 are recent paralogs

Classification of fungal Zt-KP6 like proteins

PCA analysis using the structural distances between KP6 like proteins (Z-score DALI)



7 Zt-KP6 like proteins in Zymoseptoria

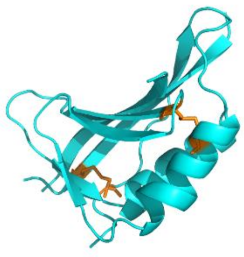
Zt-KP6-1, -2 are recent paralogs

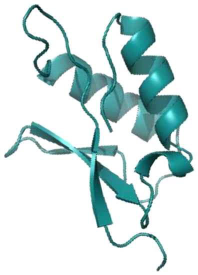
Conclusions

The structures of two effectors of *Zymoseptoria tritici*

Zt-KP4-1 and Zt-KP6-1

were characterized





Structural similarities to UmV Killer toxins suggested that these effectors were fungitoxic

This hypothesis was tested by *in vitro growth* assays : Zt-KP4 and Zt-KP6-1 have antifungal activities

Bioinformatics analysis, including Foldseek and HMM searches showed that Zt-KP4 and Zt-KP6 like proteins are widespread in fungi

Pespectives

Zt-KP4 and Zt-KP6-1 have antifungal activities
Zt-KP4-1 and Zt-KP6-1 related proteins are widespread in fungi

What are the antifungal activities of fungal effectors structurally related to Zt-KP4-1 and Zt-KP6-1 ?

Pespectives

Zt-KP4 and Zt-KP6-1 have antifungal activities
Zt-KP4-1 and Zt-KP6-1 related proteins are widespread in fungi

What are the antifungal activities of fungal effectors structurally related to Zt-KP4-1 and Zt-KP6-1 ?

Are they other fungal effectors with antimicrobial activities ?
YES: an increasing number

OXFORD FEMS

FEMS Microbiology Reviews, 2022, 1–16
DOI: 10.1093/femsre/fuac022
Advance access publication date: 23 May 2022
Review article

Microbiota manipulation through the secretion of effector proteins is fundamental to the wealth of lifestyles in the fungal kingdom

Nick C. Snelders[1,2], Hanna Rovenich[1], Bart P. H. J. Thomma[1,3,*,†]

Ref	Name	Reported activity	Organism	Organism
a015	CSαβ defensin Aclasin	Antibacterial	Fungus	Aspergillus clavatus
a016	CSαβ defensin AfusinC	Antibacterial	Fungus	Aspergillus fumigatus
a107	LYS2	Antibacterial	Fungus	Coprinopsis cinerea
a108	Copsin	Antibacterial	Fungus	Coprinopsis cinerea
a109	CPP2	Antibacterial	Fungus	Coprinopsis cinerea
a006	Efe-AfpA	Antifungal	Fungus	Epichloë festucae
a085	PAF	Antifungal	Fungus	Fusarium polyphialidicum
a086	NFAP2	Antifungal	Fungus	Neosartorya fischeri
a079	PAFC	Antifungal	Fungus	Penicillium chrysogenum
a087	pafB	Antifungal	Fungus	Penicillium chrysogenum
a043	PdAfpB	Antifungal	Fungus	Penicillium digitatum
a040	AfpA	Antifungal	Fungus	Penicillium expansum
a101	FUN_004580	Antibacterial	Fungus	Rosellinia necatrix
a102	FUN_011519	Antibacterial	Fungus	Rosellinia necatrix
r001	UhRibo1	Antibacterial	Fungus	Ustilago hordei
a050	VdAve1	Antibacterial	Fungus	Verticillium dahliae
a051	VdAve1L2	Antibacterial	Fungus	Verticillium dahliae
a052	VdAMP2	Antibacterial	Fungus	Verticillium dahliae
a053	VdAMP3	Antifungal	Fungus	Verticillium dahliae
r010	Zt6 ribonuclease	Antibacterial,Antifungal	Fungus	Zymoseptoria tritici

CSH Cold Spring Harbor Laboratory bioRxiv

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AMAPEC: accurate antimicrobial activity prediction for fungal effector proteins

Posted January 04, 2024.

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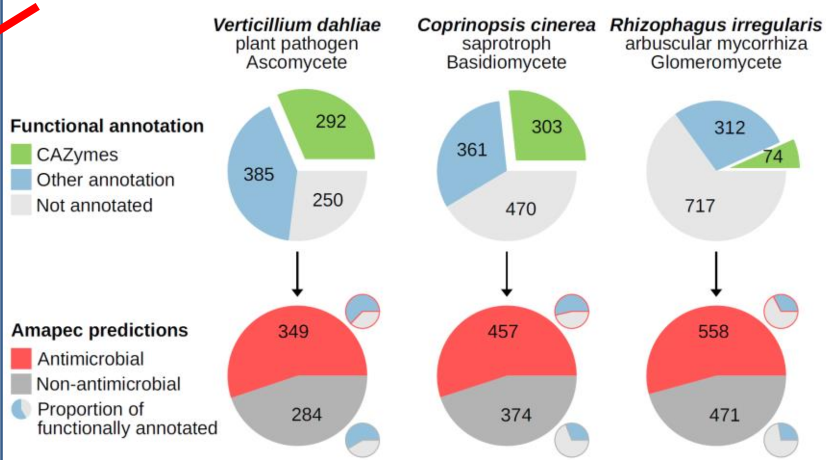
Fantin Mesny, Bart PHJ Thomma

doi: https://doi.org/10.1101/2024.01.04.574150



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PHIM

INRAE BIOGER Saclay
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Nicolas Lapalu
Justine Rouffet

BIOGER CPP

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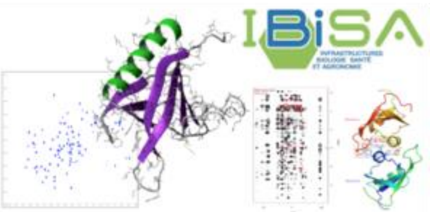
Plateau RMN



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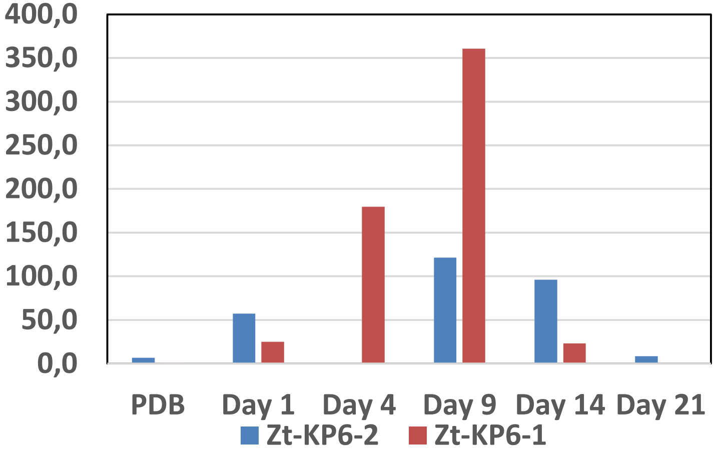
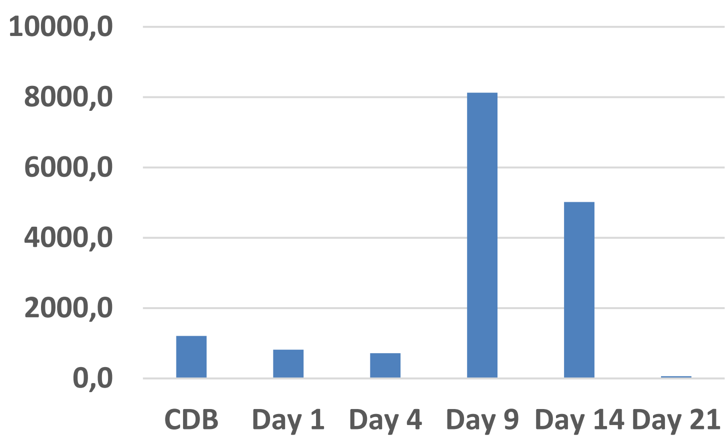
What are the possible roles of
Zt-KP4 and Zt-KP6-1
in *Z. tritici* life cycle ?

Expression of Zt-KP6-1/2 during infection



Zt-KP6-1 and 2 are mainly expressed during the switch to necrotrophy during fungal colonisation of leaves

Expression of Zt-KP4-1 during infection



Zt-KP4 is mainly expressed during the switch to necrotrophy during fungal colonisation of leaves

Same pattern as Zt-KP6-1 (20-fold higher)

Elimination of other fungi inside the leaves or on the leaf surface ?