

Functional characterisation of Nudix hydrolase effectors from phytopathogenic fungi

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University

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Declaration

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Abstract

Agricultural crop production is persistently constrained by plant-pathogenic fungi, threatening the ability of society to feed a growing global population. Central to the intricate molecular interactions occurring between plants and pathogenic fungi are small pathogen-secreted proteins (effectors). While these effectors typically exhibit vast sequence diversity due to intense evolutionary pressures, there are instances where similar effector groups are shared across multiple unrelated pathogens. The Nudix hydrolase effectors are one such group. Understanding the functions of conserved effector families offers a potential pathway towards unravelling the sophisticated interplay that occurs between multiple cropping plant species and their devastating fungal pathogens. Effector functional characterisation may also offer invaluable insights into potential strategies to improve crop disease resistance. In this thesis, I have identified and characterised effectors from pathogenic fungi that are functional Nudix hydrolase enzymes.

AvrM14, a Nudix hydrolase effector from *Melampsora lini* (flax rust), is recognised by the *Linum usitatissimum* (flax) immune receptors M1 and M4. An exhaustive substrate screen was conducted prior to my thesis, including 70 compounds often hydrolysed by Nudix hydrolases. The *in vitro* screen revealed AvrM14's remarkable substrate selectivity, the effector only hydrolysed $^{m7}Gp_5G$ (P1-(5'-7-methyl-guanosyl)-P5-(5'-guanosyl)-pentaphosphate). While $^{m7}Gp_5G$ does not occur naturally, 7-methyl guanosine (^{m7}G) is a distinctive molecular structure from the 5' cap of mRNA transcribed by eukaryotic RNA polymerase II. I conducted a sensitive *in vitro* radiolabelled assay and determined that AvrM14 can remove the protective 5' cap from mRNA and this activity requires a glutamate amino acid within the putative active site. Notably, homodimerisation of AvrM14 promoted biologically relevant mRNA cap cleavage and mRNA decapping activity is conserved in related Nudix hydrolase effectors across the *Melampsora* genus. When expressed *in planta*, AvrM14 suppresses the immune-related reactive oxygen species burst and hypersensitive cell-death response triggered by M1. Significantly, the same pivotal glutamate amino acid is indispensable both for the mRNA decapping and the immune-suppressive activities. My findings support a model whereby the Nudix hydrolase effectors from *Melampsora* spp. decap plant mRNA to suppress immune responses and ultimately promote the infection process.

Predicted Nudix hydrolase effectors also exist in *Magnaporthe oryzae* (rice blast) and multiple *Colletotrichum* spp. The Nudix effectors from *Magnaporthe* and *Colletotrichum* are more closely related to each other than the mRNA decapping effectors of *Melampsora* spp. Substrate

screening with purified effector proteins revealed that the *Magnaporthe* and *Colletotrichum* effectors hydrolyse inositol pyrophosphate signalling molecules. To understand substrate binding, I determined the crystal structure of the *M. oryzae* Nudix effector (*MoNudix*). Structural similarity searches revealed that *Homo sapiens* diphosphoinositol polyphosphate phosphohydrolase 1 (*HsDIPP1*) is remarkably similar to *MoNudix* in both overall structure and surface charge properties. *HsDIPP1* also hydrolyses inositol pyrophosphates, and using targeted mutagenesis I demonstrate that like *HsDIPP1*, *MoNudix* utilises basic amino acids to facilitate selective inositol pyrophosphate binding and hydrolysis. Inositol pyrophosphates are signalling molecules that indicate the availability of phosphate: diminished inositol pyrophosphate levels activate phosphate starvation response transcription factors, thereby driving the expression of genes responsive to phosphate deprivation. Consistent with this, the production of Nudix hydrolase effectors from *M. oryzae* and *Colletotrichum* spp. in *Nicotiana benthamiana* elevated the expression of phosphate starvation responsive genes as measured by RT-qPCR. Phosphate starvation induction was dependant on amino acids required for the hydrolysis of inositol pyrophosphates *in vitro*, and all qPCR data was corroborated using an innovative promoter/reporter system developed during the study. Work from our collaborators demonstrated that when the expression of one inositol pyrophosphate hydrolysing effector from *M. oryzae* was reduced using RNAi, there was a significant decline in the pathogen's virulence on rice. Live cell-imaging also indicates that the inositol pyrophosphate hydrolase effector is translocated into the host rice cell during infection. Collectively, the results indicate that *Magnaporthe* and *Colletotrichum* Nudix hydrolase effectors function as intracellular hydrolases, targeting inositol pyrophosphate signalling molecules which initiates plant phosphate starvation responses.

To date, the virulence functions of fungal effectors have remained largely unidentified. Through this study, I have established that the predicted Nudix hydrolase effectors from pathogenic fungi act as enzymes, likely targeting mRNA caps and inositol pyrophosphates. Collectively, my work has led to significant advances in our understanding of fungal effector function and the complex interplay occurring between pathogenic fungi and their plant hosts.

List of Abbreviations

6 x His	-	hexahistidine protein tag
ACRF	-	Australian Cancer Research Foundation
ADP	-	adenosine diphosphate
ADPR	-	adenosine diphosphate ribose
AINSE	-	Australian Institute of Nuclear Science and Engineering
AMF	-	arbuscular mycorrhizal fungi
AMP	-	adenosine monophosphate
ANOVA	-	analysis of variance
AP	-	appressoria
APIP	-	AvrPiz-t Interacting Protein
Ap _n A	-	diadenosine polyphosphate
ARC	-	Australian Research Council
ATP	-	adenosine triphosphate
AU	-	absorbance units
Avr	-	avirulence effector protein
BFA	-	brefeldin A
BIC	-	biotrophic interfacial complex
BLAST	-	basic local alignment search tool
CC	-	coiled-coil
cDNA	-	complementary DNA
CDP	-	cytidine diphosphate
CDS	-	coding sequence
CE-ESI-MS	-	capillary electrophoresis electrospray ionization mass spectrometry
CEBiP	-	chitin elicitor binding protein
CERK1	-	chitin elicitor receptor kinase 1
cNMP	-	cyclic nucleotide monophosphate
DAMP	-	damage-associated molecular pattern
DE	-	differential expression
DNA	-	deoxyribonucleic acid
dpi	-	days-post infiltration
ECD	-	extracellular domain
EIHM	-	extra-invasive hyphal membrane
ER	-	endoplasmic reticulum
ETI	-	effector-triggered immunity
FAD	-	flavin adenine dinucleotide
FDR	-	false discovery rate
GDP	-	guanosine diphosphate
GFP	-	green fluorescent protein
GMP	-	guanosine monophosphate
GO	-	gene ontology
G _p ₅ G	-	diguanosine pentaphosphate
GTP	-	guanosine triphosphate
hpi	-	hours-post inoculation
HR	-	hypersensitive response
ID	-	integrated domain
InsP ₆	-	inositol hexakisphosphate
InsP ₈	-	1/3,5-PP-InsP ₄
IQR	-	interquartile range

ITP	-	inosine triphosphate
JA-Ile	-	(3R,7S)-jasmonoyl-L-isoleucine
JGI	-	Joint Genome Institute
Kd	-	disassociation constant
kDa	-	kilodalton
LB	-	Luria-Bertani broth
LRR	-	leucine-rich repeat
LysM	-	lysin motif
^{m7} G	-	7-methyl guanosine
^{m7} Gp ₅ G	-	P1-(5'-7-methyl-guanosyl)-P5-(5'-guanosyl)-pentaphosphate
MALS	-	multi-angle light scattering
MAMP	-	microbe-associated molecular pattern
MAPK	-	mitogen-activated protein kinase
MEC	-	membranous effector compartment
mL	-	millilitre
mM	-	millimolar
MR	-	molecular replacement
mRFP	-	monomeric red fluorescent protein
mRNA	-	messenger RNA
MST	-	micro-scale thermophoresis
NAD ⁺ /NADH	-	nicotinamide adenine dinucleotide
NB	-	nucleotide binding
NCBI	-	National Centre for Biotechnology Information
ng	-	nanogram
NLR	-	nucleotide binding leucine-rich repeat receptor
nm	-	nanometre
nM	-	nanomolar
NMD	-	nonsense-mediated mRNA decay
NRD	-	N-terminal regulatory domain
ns	-	not significant
Nudix	-	nucleoside diphosphate linked to moiety-X
OD	-	optical density
ORF	-	open reading frame
p-RNA	-	5'-monophosphate RNA
P1BS	-	PHR binding sequence
PAGE	-	polyacrylamide gel electrophoresis
PCR	-	polymerase chain reaction
PDB	-	protein data bank
pEG	-	pEarleyGate
PGRA	-	Postgraduate Research Award
PHR	-	phosphate starvation response transcription factor
Pi	-	inorganic phosphate
PMSF	-	phenylmethylsulfonyl fluoride
PP-InsP	-	inositol pyrophosphate
pp-RNA	-	5'-diphosphate RNA
PPI	-	protein-protein interaction
pRib-ADP	-	2'-(5''-phosphoribosyl)-5'-adenosine diphosphate
pRib-AMP	-	2'-(5''-phosphoribosyl)-5'-adenosine monophosphate
PSI	-	phosphate starvation induced
PSR	-	phosphate starvation response

PTI	-	pattern-triggered immunity
qPCR	-	quantitative polymerase chain reaction
qRT-PCR	-	quantitative reverse transcription polymerase chain reaction
RALF	-	rapid alkalinisation factor
Rf	-	retention factor
RLCK	-	receptor-like cytoplasmic kinase
RLK	-	receptor-like kinase
RLP	-	receptor-like protein
RLU	-	relative luminescence units
RNA	-	ribonucleic acid
RNA-Seq	-	RNA-Sequencing
RNAi	-	RNA interference
ROS	-	reactive oxygen species
RTP	-	Research Training Program
S1P	-	site-1 protease
SAD	-	single-wavelength anomalous diffraction
SD	-	standard deviation
SEC	-	size-exclusion chromatography
SEM	-	standard error of the mean
T-DNA	-	transfer DNA
TB	-	terrific broth
TDP	-	thymidine diphosphate
TIR	-	toll/interleukin-1 receptor
TLC	-	thin-layer chromatography
TNT	-	tuberculosis necrotizing toxin
TPM	-	transcripts per million
tRNA	-	transfer RNA
TPP	-	thymidine triphosphate
UDP	-	uridine diphosphate
UTR	-	untranslated region
WAK	-	wall-associated-kinase
WT	-	wild-type
XTP	-	xanthosine triphosphate
Y2H	-	yeast-two hybrid
YFP	-	yellow fluorescent protein
μ L	-	microlitre

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1. Introduction

1.1 Plant diseases

In 2021, the Food and Agricultural Organisation of the United Nations estimated the global economic cost of plant diseases to be US\$220 billion annually (Fao 2021). The impact of these diseases, however, extends beyond financial losses. Recent outbreaks of coffee rust in Central America contributed to coffee farmers and labourers losing their livelihoods, leading to increased poverty and hunger (Avelino *et al.*, 2015). Notable historical events like the Irish potato famine and the Great Bengal famine underscore the catastrophic potential of crop diseases. In the Great Bengal famine alone, it is estimated that two million people died of starvation (Padmanabhan 1973). With a predicted 60% increase in global food production required by 2050 to feed the growing population (Fao 2019), society needs to detect, monitor, and control plant diseases to ensure future food security. Complicating matters further are forecasts of pathogen spread to new geographical areas prompted by changing climates (Bebber *et al.*, 2013, Bebber 2015, Delgado-Baquerizo *et al.*, 2020), the evolution of novel pathogen lineages (Singh *et al.*, 2011, Cooke *et al.*, 2012, Inoue *et al.*, 2017), and the emergence of pesticide resistance (Chen *et al.*, 2007, Castroagudín *et al.*, 2015, Dooley *et al.*, 2016). In the fight against plant pathogens, it is imperative that we understand our enemy.

1.1.1 *Melampsora* spp.

Melampsora belongs to the order *Pucciniales*, which is composed of >7000 identified species of foliar plant pathogens (Aime and Mctaggart 2021). The *Pucciniales* are commonly referred to as rust diseases, due to their characteristic rust (yellow/orange/red/brown) colouration on infected plants. Rusts sap their host plants of important nutrients, promote water loss by rapidly increasing plant transpiration during fungal sporulation, and ultimately reduce the yield of important crops (Voegele and Mendgen 2003, Grimmer *et al.*, 2012). The historical footprint of rust diseases on human society traces back over 3000 years, as indicated by archaeological evidence (Kislev, 1982). In ancient Rome, the Robigalia festival involved sacrificial offerings aimed at preventing epidemics of cereal rust diseases. Today, cereal rusts alongside other rust diseases continue to pose significant threats to agricultural productivity and are the focus of intensive characterisation and research (Singh *et al.*, 2011, Langenbach *et al.*, 2016, Moscou and Van Esse 2017, Talhinhias *et al.*, 2017, Figueroa *et al.*, 2018, Duplessis *et al.*, 2021, Petre and Duplessis 2022).

Rust fungi are obligate biotrophs, they subsist exclusively on living plant tissue. Their lifestyle is remarkably complex, with up to 5 distinct spore stages. Furthermore, many rust fungi are heteroecious, meaning they infect multiple plant host species during different stages of development (Aime *et al.*, 2017). The combination of obligate biotrophy and a complex lifestyle place rust fungi among the most difficult plant pathogens to study in a modern research environment. Therefore, to study the molecular interactions occurring during plant infection, researchers typically rely heavily on heterologous systems (reviewed in (Lorrain *et al.*, 2018)). A key player in the molecular interactions occurring between rust fungi and their host plants are small proteins secreted from the fungus called effectors, which play a pivotal role in promoting disease. However, some effectors can be recognised by host plant receptor proteins which typically leads to disease resistance (see section 1.2 for a detailed introduction to plant immunity receptors and effectors). Recognised effectors are often called Avrs (avirulence effector).

Melampsora lini is considered the ‘model’ rust species with respect to studies on effector-dependent virulence/avirulence (Lawrence *et al.*, 2007). *M. lini* is an autoecious fungus, its entire lifecycle is completed on a single host plant. Although *M. lini* infection of flax (*Linum usitatissimum*) can result in severe seed yield losses and reduced flax fibre quality, flax rust has been described as ‘more famous than infamous’ (Dean *et al.*, 2012). This description reflects the profound and fundamental knowledge studies of the flax/flax rust interaction have provided society regarding plant-microbe interactions. Harold Flor’s seminal research into the interactions between flax and flax rust in the mid-20th century (Flor 1935, 1940, 1941, 1942, 1946, 1947), mapped the ability of flax rust races to infect various flax genotypes. During this research, Flor proposed that the ‘pathogenicity of a physiologic race of *Melampsora lini* is determined by pathogenic factors specific for each resistance factor possessed by the host’. This is known as the ‘gene-for-gene’ relationship (reviewed in (Lawrence *et al.*, 2007)). The pathogenic factors have since been identified as effectors and the resistance factors as plant immunity receptors.

Over the past few decades various avirulence effectors from flax rust and their cognate receptor proteins in flax have been identified (Lawrence *et al.*, 1995, Anderson *et al.*, 1997, Ellis *et al.*, 1999, Dodds *et al.*, 2001, Dodds *et al.*, 2004, Catanzariti *et al.*, 2006, Lawrence *et al.*, 2010) and their interactions characterised (Catanzariti *et al.*, 2010, Ravensdale *et al.*, 2012, Ve *et al.*, 2013, Anderson *et al.*, 2016, Zhang *et al.*, 2018). These studies have revealed important details regarding the recognition of Avrs. However, a comprehensive understanding of how rust effectors promote virulence in the absence of recognition remains elusive. The presented thesis

will provide an in-depth analysis into how a group of *Melampsora* effectors act as enzymes to promote virulence. Understanding the virulence activities of rust effectors is essential for a comprehensive understanding rust disease.

1.1.2 *Magnaporthe* spp.

Species within the *Magnaporthe* (*syn. Pyricularia*) genus are the causal agents of blast disease afflicting various monocot grass species, including vital crops such as rice, wheat, barley, and finger millet. Of particular concern to the global economy and food security is *Magnaporthe oryzae*, which restrains rice yield (Talbot 2003, Fernandez and Orth 2018), can devastate finger millet crops (Mbinda and Masaki 2020), and poses an emerging threat to wheat production (Cruz and Valent 2017, Ceresini *et al.*, 2018, Ceresini *et al.*, 2019). Annually, this one fungal species is responsible for the destruction of enough food to sustain hundreds of millions of people (Fisher *et al.*, 2012). *M. oryzae* can be separated into several host-adapted lineages, including *Oryza* (rice), *Triticum* (wheat), *Setaria* (foxtail millet), *Eleusine* (finger millet), and *Lolium* (ryegrass) lineages (Gladieux *et al.*, 2018). Isolates within each lineage are typically associated with aggressive blast disease on their preferred host plant, although there are instances of isolates straying beyond their typical lineages to target alternative hosts (Farman *et al.*, 2017, Gladieux *et al.*, 2018). Concerningly, *M. oryzae* is poised for further host expansion and specialisation (Valent *et al.*, 2019). The combination of gene flow between lineages (Gladieux *et al.*, 2018), the ability to infect multiple hosts (Mackill and Bonman 1986, Chung *et al.*, 2020, Paul *et al.*, 2022), and the proximity in which various *M. oryzae* hosts often grow, could expedite the emergence of novel and aggressive *M. oryzae* strains. This threat is exemplified by the recent development of wheat blast disease, which was first reported in Brazil in 1985 (Igarashi 1986) and spread to Bangladesh in 2016 (Surovy *et al.*, 2023) and Zambia in 2017 (Tembo *et al.*, 2020).

M. oryzae is a hemibiotrophic pathogen, initially extracting nutrients from living host tissue before causing plant cell death. Detailed analysis of the rice infection process has revealed that infection begins by three-celled spores (conidia) actively attaching to the surface of a leaf (Hamer *et al.*, 1988). These conidia germinate and extend a germ tube across the leaf surface (Figure 1). An infection structure called an appressorium forms at the tip of the germ tube; the appressorium generates extreme turgor pressure (up to 8.0 MPa) (Howard *et al.*, 1991) to drive a penetration peg through the host cuticle, breaching the epidermal cells beneath (Dagdas *et al.*, 2012). In a successful infection, *M. oryzae* develops intimate connections with living plant

cells (Figure 1). Live-cell imaging suggests that *M. oryzae* transits between plant cells by penetrating pit fields, where plasmodesmata cluster (Kankanala *et al.*, 2007, Sakulkoo *et al.*, 2018) (Figure 1). *M. oryzae* interacts with invaded rice cells using intracellular hyphae sealed within a plant-derived membrane, named the extra-invasive hyphal membrane (EIHM) (Kankanala *et al.*, 2007) (Figure 1). A dome-shaped region of the EIHM, the biotrophic interfacial complex (BIC), is the site of effector translocation from the fungus into the host cell (Khang *et al.*, 2010) (Figure 1). The functions of most *M. oryzae* effectors are unknown, although some have demonstrated roles interfering with plant immunity (Park *et al.*, 2012, Kim *et al.*, 2020), and preventing pathogen recognition (Mentlak *et al.*, 2012). Identifying how *M. oryzae* effectors promote intracellular colonisation will provide a more comprehensive understanding of this critically important fungal species and may direct the development of cropping plants with enhanced blast disease resistance.

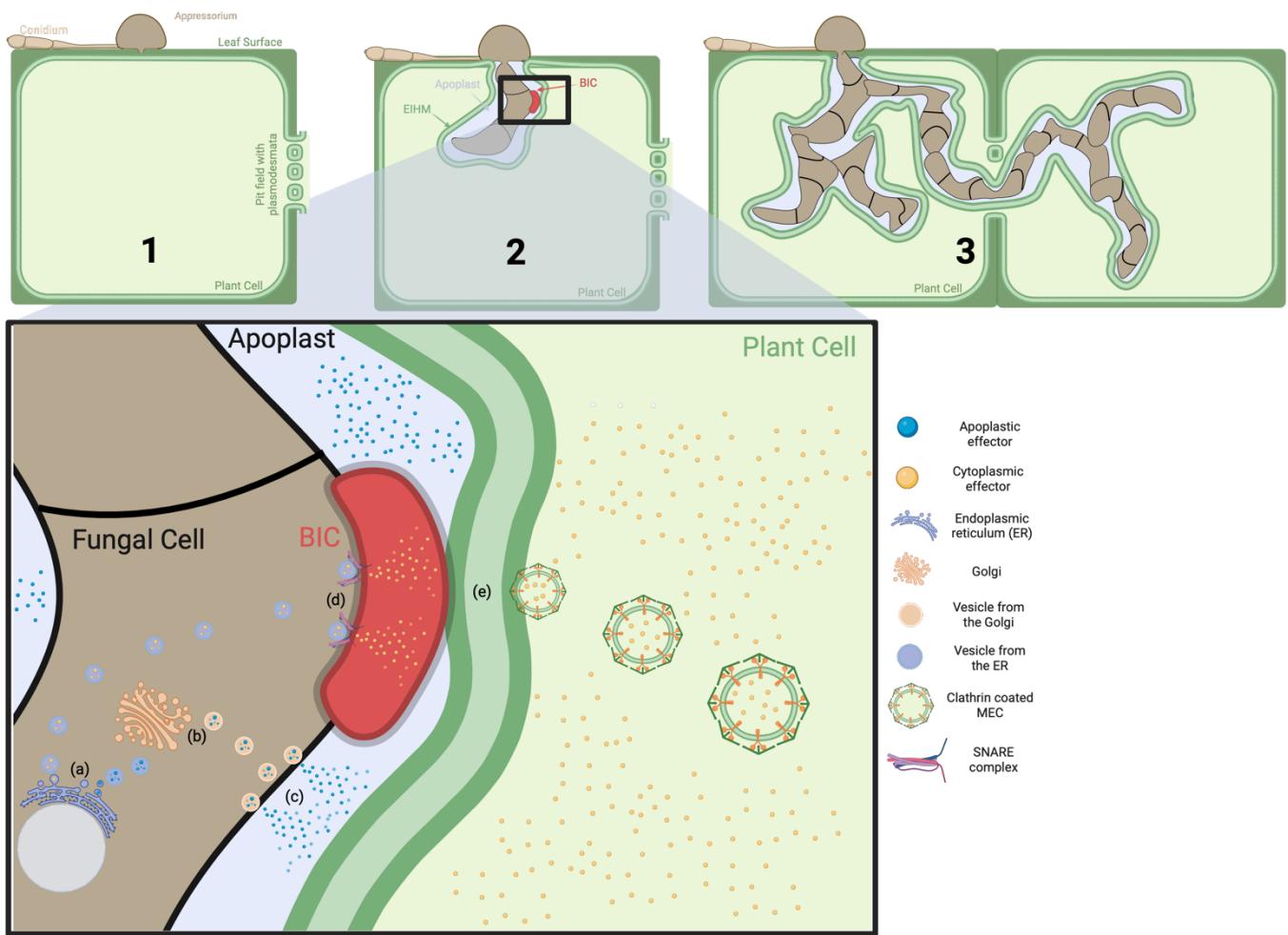


Figure 1: The rice blast infection process and *Magnaporthe oryzae* effector secretion pathways. (Top) (1) Infection begins when a fungal spore (conidium) attaches to the surface of a leaf. The spore extends a germ tube across the leaf surface, at the tip of the germ tube an appressorium is formed. The appressorium generates extreme turgor pressure to drive a penetration peg through the host cuticle and into the epidermal plant cells beneath. (2) A fungal hypha grows into the breached plant cell. Fungal hyphae are sealed within a plant-derived membrane, named the extra-invasive hyphal membrane (EIHM). The region between the fungal cell wall and the plant plasma membrane is known as the apoplast. A dome-shaped region of the EIHM, the biotrophic interfacial complex (BIC), is the site of effector translocation from the fungus into the host cell. (3) As the infection progresses, *M. oryzae* hyphae transit between plant cells by penetrating pit fields, where the cell wall is thinner and where plasmodesmata cluster. (Bottom) Apoplastic and cytoplasmic effectors are secreted by distinct mechanisms in *M. oryzae*. (a) The translation of apoplastic and cytoplasmic effectors may occur in divergent subcellular environments, as only cytoplasmic effector expression is sensitive to changes in ribosomal pausing during codon decoding by tRNAs. (b and c) Apoplastic effectors are secreted into the apoplast from the endoplasmic reticulum via a Golgi-dependant mechanism. (d) The translocation of cytoplasmic effectors into the BIC requires the exocyst complex including SNAREs. (e) To cross the plant membrane at the BIC, cytoplasmic effectors are packaged into clathrin-coated vesicle-like membranous effector compartments (MECs) derived from plant membranes.

1.2 Molecular plant immunity

To detect pathogens and initiate immune responses, plants rely on receptor proteins at the cell surface and within the cell (Kanyuka and Rudd 2019, Van Der Burgh and Joosten 2019, Mccombe *et al.*, 2022). The receptors sense molecules characteristic of pathogen infection, including microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPs), plant-derived immunomodulatory peptides called phytocytokines, and effectors. The responses activated by plant receptors can include the production of reactive oxygen species (ROS) and anti-microbial compounds, the influx of Ca^{2+} into the cytosol, immune signalling via mitogen-activated protein kinase (MAPK) cascades, transcriptional reprogramming, and a hypersensitive response (HR) leading to localised plant cell death (Tsuda and Katagiri 2010, Peng *et al.*, 2018). To undermine and overcome the innate plant immune system, pathogens utilise effectors.

1.2.1 Pathogen recognition

The apoplast, the region situated between the fungal cell wall and plant plasma membrane, can be monitored by plant cells to enable pathogen detection early during infection. Apoplastic pathogen recognition is controlled by a network of receptors embedded in the plant plasma-membrane. Most cell-surface pathogen-recognition receptors are categorised as either receptor-like proteins (RLPs) or receptor-like kinases (RLKs), depending on the absence or presence of an intracellular kinase domain. Both receptor classes exhibit a membrane-embedded domain and an extracellular ligand-binding domain. RLPs and RLKs can be further classified into subfamilies based on their extracellular domain (ECD). Identified ECDs of plant pathogen recognition receptors include lysin motif (LysM), leucine-rich repeat (LRR), lectin, epidermal growth factor-like, galacturonan-binding wall-associated-kinase (WAK) and WAK-associated C-terminal domains (Macho and Zipfel 2014, Stephens *et al.*, 2022). In the fight against fungal pathogens, LysM-RLP/RLKs are crucial as they can detect chitin oligomers released from the cell-wall of invading fungi. Chitin elicitor receptor kinase 1 (CERK1) is a LysM-RLK involved in chitin-induced defense responses and is present in both monocot and dicot plants (Miya *et al.*, 2007, Shimizu *et al.*, 2010). In rice, CERK1 is recruited by the chitin elicitor binding protein (CEBiP), a LysM-RLP, following homodimerisation of CEBiP promoted by binding to long-chain chitin oligomers (Hayafune *et al.*, 2014). The CERK1 and CEBiP complex activates immune signalling pathways and ultimately promotes fungal disease resistance (Hayafune *et al.*, 2014). Rice CERK1 is also implicated in symbiosis with arbuscular

mycorrhizal fungi (AMF) (Miyata *et al.*, 2014, Zhang *et al.*, 2015) and can also detect short-chain chitin oligomers produced by AMF when in complex with MYR1 (another LysM-RLK) (Carotenuto *et al.*, 2017, He *et al.*, 2019). A recent discovery demonstrated that MYR1/CERK1 complex formation reduces the chitin-induced immune response by inhibiting the recruitment of CERK1 by CEBiP (Zhang *et al.*, 2021). By recognising highly conserved fungal cell wall components, LysM-RLPs/RLKs can inhibit the growth of a diverse range of pathogenic fungi, including biotrophs like *Melampsora spp.*, and hemibiotrophs like *Magnaporthe spp.*, while also supporting interactions with beneficial fungi.

In addition to detecting wide-spread and conserved fungal molecules like chitin and other MAMPs, plants can detect rapidly evolving and diverse effector proteins secreted by pathogenic fungi. While there are examples of apoplastic fungal effectors being recognised by extracellular receptors (Jones *et al.*, 1994, Dixon *et al.*, 1996, Thomas *et al.*, 1997, Dixon *et al.*, 1998, Takken *et al.*, 1999, Kawchuk *et al.*, 2001, De Jonge *et al.*, 2012), most identified receptors for rust and blast Avrs are cytosolic nucleotide binding (NB) leucine-rich repeat (LRR) receptors (NLRs) (Periyannan *et al.*, 2017, Wang *et al.*, 2017). Typically, NLRs are composed of a C-terminal LRR domain, central nucleotide-binding (NB) domain, and variable N-terminal domain. The two major classes of NLR are CC-NLRs that have a coiled-coil N-terminal domain and TIR-NLRs with a toll/interleukin-1 receptor N-terminal domain. In general, the CC and TIR domains are required for immune signalling following activation, and the NB and LRR domains regulate NLR activation. There are 6 *M. lini* avirulence effectors identified that are recognised by 19 flax NLRs. Interestingly, all identified flax NLRs that recognise flax rust effectors are TIR-NLRs. In contrast, monocot species like rice lack TIR-NLRs (Shao *et al.*, 2016). In rice, most identified *M. oryzae* effector recognising receptors are CC-NLRs, with some containing additional integrated domains (IDs) required for effector recognition (reviewed in (Marchal *et al.*, 2022)). The activation of an NLR by fungal effector recognition often results in a HR that prevents the growth of biotrophic and hemibiotrophic pathogens (Balint-Kurti 2019).

1.2.2 Plant immune signalling

Upon recognition of MAMPs/DAMPs/phytocytokines, extracellular receptors initiate a series of immune responses broadly classified as pattern-triggered immunity (PTI). Our knowledge of PTI is mostly informed by studies on the bacterial MAMP flg-22 (a small peptide from bacterial flagellin). However, there is clear overlap between the PTI signalling pathways

activated by bacterial and fungal MAMP recognition (Nühse *et al.*, 2000, Zhang *et al.*, 2010, Kadota *et al.*, 2014). In all characterised examples, elicitor binding triggers an oligomerisation event that brings the intracellular domains of multiple membrane-bound receptor proteins into proximity (Chinchilla *et al.*, 2007, Liu *et al.*, 2012, Sun *et al.*, 2013, Tang *et al.*, 2015). The activated receptors recruit members of the receptor-like cytoplasmic kinase (RLCK) subfamily and participate in intricate auto- and/or trans-phosphorylation events of the intracellular receptor domains and RLCKs to facilitate downstream signalling (Lu *et al.*, 2010, Petutschnig *et al.*, 2010, Liu *et al.*, 2013, Cao *et al.*, 2014, Shinya *et al.*, 2014, Kong *et al.*, 2016, Suzuki *et al.*, 2016, Yamada *et al.*, 2016, Erwig *et al.*, 2017, Lal *et al.*, 2018). BIK1 is a well-studied and central *Arabidopsis thaliana* RLCK that integrates signals from multiple extracellular receptors, including those detecting diverse bacterial, fungal, and plant derived molecules (Lu *et al.*, 2010, Zhang *et al.*, 2010, Liu *et al.*, 2013). Following phosphorylation events, BIK1 undergoes monoubiquitination prior to release from the receptor complex (Ma *et al.*, 2020). BIK1 then goes on to directly regulate ROS generation (Kadota *et al.*, 2014, Li *et al.*, 2014), calcium ion channel activation (Tian *et al.*, 2019), and hormone production (Lal *et al.*, 2018). Immune-related ROS generation is primarily controlled by the plasma membrane-localised NADPH oxidase RBOHD (Nühse *et al.*, 2007, Zhang *et al.*, 2007). BIK1 interacts with and phosphorylates RBOHD to activate ROS signalling (Kadota *et al.*, 2014, Li *et al.*, 2014). ROS have been proposed to possess a multitude of functions in promoting plant immunity, including acting as antimicrobial compounds (Peng and Kuc 1992), regulating stomatal closure (Macho *et al.*, 2012), catalysing reactions involved in cell wall remodelling (Brown *et al.*, 1998), and acting as important secondary messenger molecules to trigger further immune responses (Wu *et al.*, 2023). One mechanism by which ROS transduce signalling is via oxidation of plant proteins. For example, a cysteine amino acid in the thiol peroxidase protein PRXIIB is oxidised by ROS produced during plant immune signalling (Bi *et al.*, 2022), the oxidised PRXIIB cysteine subsequently conjugates with a free cysteine on the protein serine/threonine phosphatase ABSCISIC ACID-INSENSITIVE2 (ABI2). The conjugation of PRXIIB and ABI2 reduces the phosphatase activity of ABI2 and promotes stomata-mediated immunity against the bacterial pathogen *Pseudomonas syringae* (Bi *et al.*, 2022).

Effector recognition by NLRs also involves protein oligomerization events, prior to signal transduction and the activation of immune responses known as effector-triggered immunity (ETI) (Wang *et al.*, 2019, Li *et al.*, 2020, Ma *et al.*, 2020, Martin *et al.*, 2020, Förderer *et al.*, 2022, Zhao *et al.*, 2022, Contreras *et al.*, 2023). The recent structures of two CC-NLRs, ZAR1

from *A. thaliana* and Sr35 from wheat, demonstrate that the activated receptors form a pentameric complex (termed resistosome) with a characteristic funnel shaped structure that associates with the plant plasma membrane (Wang *et al.*, 2019, Förderer *et al.*, 2022, Zhao *et al.*, 2022). Current models indicate that CC-NLR resistosomes function as calcium-ion channels that cause an influx of Ca^{2+} into the cytosol to trigger immune signalling that culminates in cell death (Bi *et al.*, 2021, Jacob *et al.*, 2021, Förderer *et al.*, 2022). TIR-NLRs also form oligomeric complexes following effector recognition. The structures of two TIR-NLRs, ROQ1 from *Nicotiana benthamiana* and RPP1 from *A. thaliana*, demonstrate tetrameric complex formation (Ma *et al.*, 2020, Martin *et al.*, 2020). The TIR-NLR tetramers revealed binding sites for small nucleotide molecules, consistent with the ability of multiple TIR-domains to hydrolyse NAD^+ (nicotinamide adenine dinucleotide) into ADPR (adenosine diphosphate ribose) and cyclic ADPR isomers (Horsefield *et al.*, 2019, Wan *et al.*, 2019). Recent advances have identified that TIR-NLRs can also use NAD^+ and ATP to produce multiple nucleotide molecules including pRib-AMP, pRib-ADP, ADPR-ATP, and di-ADPR (Huang *et al.*, 2022, Jia *et al.*, 2022). For *A. thaliana* and *N. benthamiana*, TIR-catalysed nucleotide molecules can function as signals that ultimately lead to the activation of CC-NLR NRG1 and calcium-ion channel formation (Qi *et al.*, 2018, Jacob *et al.*, 2021). It is notable that plant TIR domains can also produce cyclic nucleotide monophosphates (cNMPs) from nucleic acid, which are required for TIR-NLR mediate plant cell death (Yu *et al.*, 2022). However, how cNMPs integrate with the other TIR-catalysed small molecule signalling and induce plant immune responses requires clarification (Essuman *et al.*, 2022).

Both PTI and ETI responses ultimately converge on similar cellular responses (Jacob *et al.*, 2018), albeit often with differences in amplitude (Yuan *et al.*, 2021, Bernoux *et al.*, 2022). One example is the intracellular influx of Ca^{2+} , which occurs in tightly controlled bursts during PTI but in a long, sustained influx during ETI (Kim *et al.*, 2022). PTI and ETI are also intertwined, with the two pathways requiring and potentiating each other (Ngou *et al.*, 2021, Pruitt *et al.*, 2021, Tian *et al.*, 2021, Yuan *et al.*, 2021, Lang *et al.*, 2022). Overall, the plant immune system is incredibly successful at recognising incoming threats and halting infection. To overcome the host immune system and facilitate colonisation, adapted pathogens rely on effector proteins that often interfere with PTI and ETI.

1.2.3 Overcoming plant defences with effectors

Fungal effectors are generally small proteins (< 300 amino acids), have signal peptides to ensure translocation out of the fungal cell, and often demonstrate positive evolutionary selection pressures (Joly *et al.*, 2010, Stukenbrock *et al.*, 2011, Wicker *et al.*, 2013, Menardo *et al.*, 2017, Beckerson *et al.*, 2019). The rapid evolution of effectors likely reflects the need to prevent recognition by plant immune receptors while continuing to optimise virulence function (Lo Presti *et al.*, 2015). Effectors can be broadly split into two groups, those localised to the apoplast and the effectors that move across the plant plasma membrane into the plant cell cytoplasm. Recent progress in predicting fungal effector localisation using machine-learning models, suggests that approximately two-thirds of all *M. lini* effectors and over half of all *M. oryzae* effectors translocate into the host plant cell cytoplasm (Sperschneider and Dodds 2022). Fungal effector secretion systems and the mechanisms differing between cytoplasmic and apoplastic effector secretion are perhaps best characterised in the *M. oryzae*-rice pathosystem. In *M. oryzae*, apoplastic effectors travel through the endoplasmic reticulum (ER) to the Golgi prior to secretion (Giraldo *et al.*, 2013) (Figure 1). Treatment with brefeldin A (BFA), a potent inhibitor of Golgi trafficking (Chardin and McCormick 1999), prevents the secretion of apoplastic *M. oryzae* effectors (Giraldo *et al.*, 2013). These data indicate that apoplastic effectors follow the well-studied conventional protein secretion pathway, involving signal-peptide dependant co-translational insertion of the nascent polypeptide chain into the ER membrane, trafficking through the ER and vesicle-mediated transport to the Golgi, protein sorting at the Golgi, and finally transport to the surface of the fungal cell (Rodrigues *et al.*, 2013) (Figure 1). In contrast, cytoplasmic effector secretion is BFA-insensitive and therefore Golgi-independent (Giraldo *et al.*, 2013). Furthermore, recent evidence indicates that cytoplasmic and apoplastic effectors from *M. oryzae* are translated in different sub-cellular environments, as cytoplasmic but not apoplastic effectors are sensitive to changes in ribosomal pausing during codon decoding by tRNAs (Li *et al.*, 2023). After translation, the secretion of cytoplasmic effectors to the BIC requires components of the fungal exocyst complex (Giraldo *et al.*, 2013) (Figure 1). To then cross the plant membrane at the BIC, cytoplasmic effectors are packaged into vesicle-like membranous effector compartments (MECs) derived from plant membranes and lined with CLATHRIN LIGHT CHAIN 1 (Oliveira-Garcia *et al.*, 2023), indicating that *M. oryzae* co-opts plant clathrin-mediated endocytosis pathways to facilitate the uptake of cytoplasmic effectors (Figure 1). While most rice blast and rust effectors likely localise within the host cytoplasm, our knowledge of their functions remains limited.

For rust fungi, the best-characterised cytoplasmic effectors are AvrL567 and AvrP. Yeast two-hybrid (Y2H) screens identified *LuCKX1.1* as a potential target of AvrL567 (Wan *et al.*, 2019). *LuCKX1.1* is a member of the cytokinin oxidase/dehydrogenase family, enzymes responsible for the inactivation of cytokinin phytohormones (Schmülling *et al.*, 2003). In *in vitro* enzymatic assays, the incubation of *LuCKX1.1* with AvrL567 enhances *LuCKX1.1* activity, suggesting that AvrL567 may function by inhibiting cytokinin signalling (Wan *et al.*, 2019). However, no changes could be detected in cytokinin levels in whole flax plants overexpressing AvrL567 (Wan *et al.*, 2019). In flax rust, knock out of AvrL567 did not reduce pathogen virulence, indicating that either AvrL567's activity only makes a very minor or no contribution to rust virulence, or that there are functionally redundant effectors which make up for the loss of AvrL567 (Lawrence *et al.*, 2010). For AvrP, the protein's crystal structure revealed three zinc ions co-ordinated by cysteine and histidine amino acids (Zhang *et al.*, 2018). Confocal microscopy analysis of *N. benthamiana* leaves expressing fluorescent-protein tagged AvrP identified a mostly nuclear localisation, despite the absence of a nuclear localisation signal (Zhang *et al.*, 2018). The localisation of AvrP may be influenced by interactions with flax proteins. Y2H experiments identified three flax proteins (a phosphoglucomutase, peptidyl-prolyl cis-trans isomerase, and DEAD-box ATP-dependent RNA helicase) capable of interacting with AvrP (Farah 2018). All AvrP interactions could be reconstituted in *N. benthamiana* plants using bimolecular fluorescence complementation (Akram 2023). Using purified recombinant proteins, *in vitro* experimentation demonstrates that AvrP can modulate the activity of a flax phosphoglucomutase to increase the conversion of glucose-1-phosphate to glucose-6-phosphate (Akram 2023). Furthermore, AvrP can inhibit the ATPase and helicase activity of a flax DEAD-box ATP-dependent RNA helicase and expression of AvrP in flax results in major transcriptional changes. The initial characterisation of AvrP suggests it is a multifunctional effector with diverse host targets (Akram 2023).

M. oryzae effector AvrPiz-t also interacts with multiple intracellular host proteins, with 12 putative interactors identified in Y2H screens named APIP1-12 (AvrPiz-t Interacting Protein 1-12) (Park *et al.*, 2012). AvrPiz-t can be ubiquitinated by and suppresses the ubiquitin ligase activity of APIP6 and APIP10, two rice RING E3 ubiquitin ligases (Park *et al.*, 2012, Park *et al.*, 2016). AvrPiz-t can also bind to the rice plasma membrane K⁺-channel protein OsAKT1 (APIP7), interfering with the association between OsAKT1 and the cytoplasmic kinase OsCIPK23, leading to reduced K⁺ influx into the plant cell (Shi *et al.*, 2018). Interestingly, two of the AvrPiz-t interactors are involved in the recognition of AvrPiz-t and activation of ETI by the NLR Piz-t (Park *et al.*, 2016, Wang *et al.*, 2016). APIP10 promotes the degradation of Piz-

t, and therefore AvrPiz-t-mediated suppression of ubiquitin ligase activity promotes the accumulation of Piz-t. Likewise, APIP5, a bZIP-type transcription factor, also interacts with Piz-t to protect against the virulence activities of AvrPiz-t (Wang *et al.*, 2016). It is remarkable that such a small protein, comprising 90 amino acids after signal peptide removal, can interact with and modulate the activity of various plant proteins with diverse sequences. The findings from AvrPiz-t suggest that the currently uncharacterised approximately 500 predicted *M. oryzae* effectors (Sperschneider and Dodds 2022) could target over 6000 unique plant processes. However, this number is likely much smaller, as many effectors are predicted to be functionally redundant (Giraldo and Valent 2013, Lanver *et al.*, 2017). Functional redundancy, where multiple effectors can perform the same function, is often cited as the reason for the observed lack of virulence phenotypes in effector gene knockout experiments. For instance, in a large-scale single-gene knockout screen of predicted *M. oryzae* effectors, only one out of the 78 tested effectors significantly contributed to pathogen virulence on the susceptible rice cultivar Shin No. 2 (Saitoh *et al.*, 2012). Functional redundancy makes characterising the importance of a specific virulence function for pathogenicity difficult. To expedite the identification and characterisation of important effector virulence functions in multiple plant pathogen interactions, it could be advantageous to focus on effector families with conserved functions across diverse pathosystems. This thesis delves into the virulence functions of the Nudix hydrolase effector families in multiple pathogenic fungi, including in *M. lini* and *M. oryzae*.

1.3 Nudix hydrolase effectors

The Nudix (**nucleoside diphosphate linked to moiety-X**) hydrolase superfamily is ubiquitous in prokaryotic and eukaryotic organisms. These enzymes play key roles in diverse physiological processes and in the maintenance of cellular homeostasis (Mclennan 2006). Nudix hydrolases typically function as pyrophosphohydrolase enzymes, catalysing the hydrolysis of pyrophosphate bonds. The highly conserved Nudix-box consensus sequence: GX5EX7REUXEEXGU (Figure 2A) is required for Nudix hydrolase activity. In this sequence, 'U' typically represents a hydrophobic residue, while 'X' can be any amino acid (Mclennan 2006). The first glutamate and arginine form a stabilising salt bridge between a flexible loop region and α -helix, and the remaining glutamates are generally responsible for divalent metal cation binding (Figure 2A); most Nudix enzymes are hypothesised to bind to Mg^{2+} in physiological environments (Mclennan 2006). The metal cations bind to the pyrophosphate of

the substrate, neutralising and orienting it towards a general base. This base, often a glutamate or aspartate, extracts a proton from H₂O, producing a free hydroxide ion. The nucleophilic hydroxide ion hydrolyses the bound pyrophosphate (Mildvan *et al.*, 2005). Nudix hydrolases can act on several well-characterised substrates. These include intact and oxidatively damaged nucleoside triphosphates (Sakumi *et al.*, 1993, Ito *et al.*, 2005, Jemth *et al.*, 2018), ADPR (O'handley *et al.*, 1998, Gabelli *et al.*, 2002, Kang *et al.*, 2003, Perraud *et al.*, 2003), diadenosine polyphosphates (Ap_nAs) (Bessman *et al.*, 2001, Leslie *et al.*, 2002, Iwai *et al.*, 2004), and the 5' cap of mRNA transcripts (Van Dijk *et al.*, 2002, Wang *et al.*, 2002, Song *et al.*, 2013). Despite their diverse range of substrates and sequences, all structurally characterised Nudix hydrolases possess the Nudix fold with an approximately 130 amino acid α - β - α sandwich domain architecture (Srouji *et al.*, 2017) (Figure 2B). The Nudix fold is also present in isopentenyl diphosphate isomerasases and adenine glycosylases, although these families notably lack the Nudix motif sequence (Srouji *et al.*, 2017).

Nudix hydrolase domains have been predicted in effectors from multiple fungal, oomycete, and bacterial plant pathogens (Dong and Wang 2016). The putative Nudix hydrolase effectors were identified through their similarities with the Nudix-box consensus sequence (Figure 2A). Experimentally, a subset of these Nudix effectors have been confirmed to hydrolyse pyrophosphate bonds in substrates containing nucleosides.

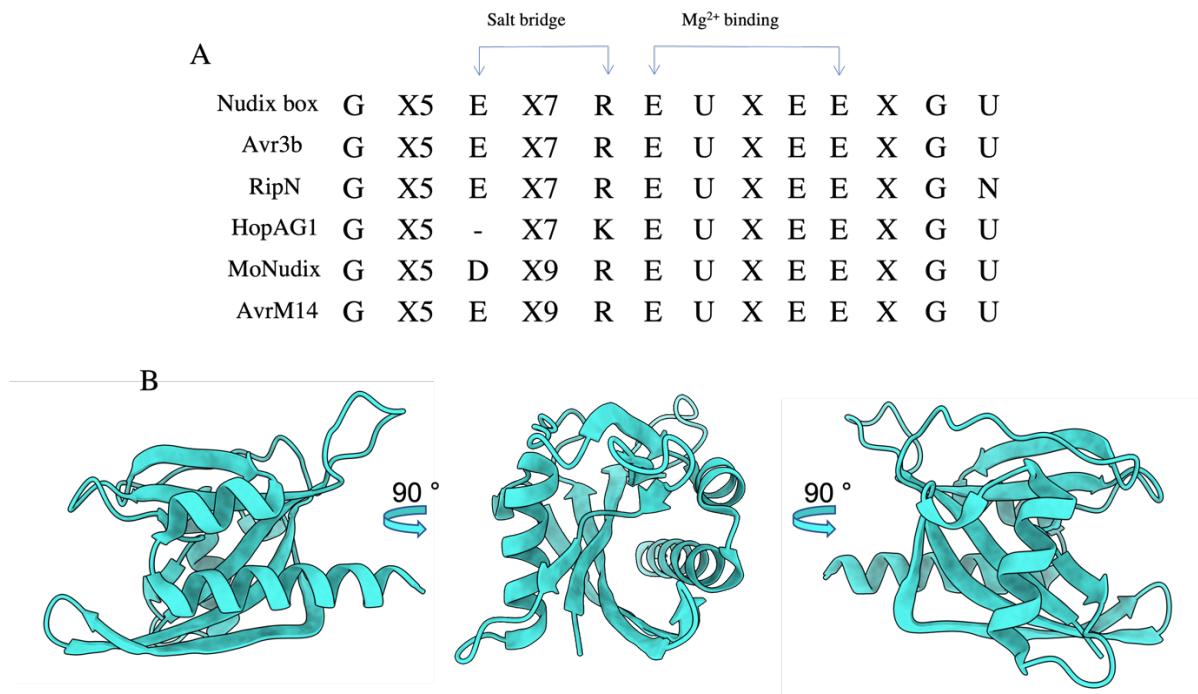


Figure 2: Characteristic features of Nudix hydrolase enzymes and Nudix box sequences from predicted effectors. (A) The conventional Nudix box consensus sequence (top) and the putative Nudix box sequences identified in effectors from phytopathogens. The glutamate and arginine which typically form a stabilising salt bridge, and the glutamates often involved in Mg²⁺ binding, are indicated with arrows. (B) The α-β-α sandwich Nudix fold, the example shown is from *Homo sapiens* Nudix hydrolase 2 (PDB ID: 3U53) (Ge *et al.*, 2013).

1.3.1 Avr3b

Avr3b from the oomycete *Phytophthora sojae*, a destructive soybean pathogen, was the first Nudix hydrolase effector to be experimentally validated. The mature Avr3b protein consists of 297 amino acids and can be recognised by soybean cultivars with the Rps3b resistance gene (Dong *et al.*, 2011). Pathogen strains virulent on Rps3b-soybean display multiple polymorphisms in their Avr3b gene. The polymorphisms include a premature stop codon that truncates 85 amino acids from the c-terminus of the protein, preventing recognition by Rps3b (Dong *et al.*, 2011). When Avr3b is transiently expressed in *N. benthamiana*, it increases susceptibility to *Phytophthora capsica* and *Phytophthora parasitica* infection. Avr3b also reduces ROS accumulation around invasion sites and suppresses plant cell death during infection (Dong *et al.*, 2011). In soybean, the expression of Avr3b suppresses cell-death triggered by the recognition of Avr1b (Dong *et al.*, 2011). For enzymatic activation, Avr3b requires a plant cyclophilin which likely catalyses the isomerisation of a proline in the Avr3b protein. Both the cyclophilin-targeted proline and the Nudix-box are essential for the observed virulence activities of Avr3b (Dong *et al.*, 2011, Kong *et al.*, 2015). Avr3b has been tested with a limited range of substrates and was able to hydrolyse NADH and ADPR (Dong *et al.*, 2011).

Notably, even though the truncated form of Avr3b is not recognised by Rps3b, it still maintains its Nudix hydrolase activity. There are also related Nudix effectors in other *Phytophthora* spp. as well as in *Hyaloperonospora parasitica*, another phytopathogenic oomycete (Dong and Wang 2016). Recently, Yu *et al.*, (2022) demonstrated that Avr3b also has cyclic nucleotide phosphodiesterase activity against the cNMPs produced by plant TIR-domains and suggest that this enzymatic activity is a virulence function of the effector. The study also identified *A. thaliana* Nudix hydrolase 7 (*AtNudx7*) as a plant cNMP phosphodiesterase (Yu *et al.*, 2022). *AtNudx7* is a well-known negative regulator of EDS1-dependant immune signalling, a pathway which is activated by the small nucleotide molecules produced by TIR-NLRs (Bartsch *et al.*, 2006, Ge *et al.*, 2007, Huang *et al.*, 2022, Jia *et al.*, 2022). Yu *et al* (2022) propose that *AtNudx7* negatively regulates TIR-NLR signalling by hydrolysing cNMPs. Alternatively, other *in vitro* substrates for *AtNudx7* have been identified previously (including ADPR, NADH, NAD⁺, AP₃A, AP₄A, AP₅A, FAD, UDP-glucose, and ADP-glucose) (Ogawa *et al.*, 2005, Olejnik and Kraszewska 2005) and these substrates share greater structural similarities with other TIR-produced and pyrophosphate-containing immunomodulatory molecules such as di-ADPR, ADPR-ATP, and pRib-ADP. The promiscuous *in vitro* enzymatic activity of both *AtNudx7* and Avr3b is a trait often observed in Nudix hydrolases (McLennan 2013) and complicates the identification of *in vivo* substrates.

1.3.2 RipN

The pathogenic bacterium *Ralstonia solanacearum* is responsible for bacterial wilt disease in various crops, including potato, tomato, and banana. *R. solanacearum* secretes RipN, a 473-amino acid Nudix hydrolase effector that hydrolyses a variety of nucleoside diphosphate substrates *in vitro* (Sun *et al.*, 2019). Similar to *AtNudx7* and Avr3b, the preferred *in vitro* substrates of RipN are NADH and ADPR; however, overexpression of RipN in *A. thaliana* did not result in significant changes to ADPR, NAD⁺ or NADH levels (Sun *et al.*, 2019). Unlike Avr3b, it is unlikely that RipN mimics the activity of *AtNudx7* to negatively regulate plant immunity. This is because RipN localises to the nuclear and endoplasmic reticulum membranes when expressed in *N. tabacum* and *A. thaliana* with a GFP tag, whereas GFP-tagged *AtNudx7* localises in the aqueous cytoplasm/nucleus (Olejnik *et al.*, 2011, Sun *et al.*, 2019). The localisation of RipN does not require the Nudix domain and is dependent on the c-terminus, which has no homology to proteins of known function or identifiable localisation sequences (Sun *et al.*, 2019). Transgenic *A. thaliana* plants expressing RipN demonstrate compromised

PTI, including reduced defence gene-induction and callose deposition following treatment with flg-22. RipN with a mutated Nudix box was unable to interfere with PTI, indicating the importance of Nudix hydrolase activity (Sun *et al.*, 2019). The large size of RipN suggests that the Nudix box is not the only functional region in the effector, perhaps identifying protein interactors will provide further insight into the virulence function/s of RipN.

1.3.3 HopAG1

HopAG1 (previously known as HolPsyAG) was identified as a potential Nudix effector in *Pseudomonas syringae* pv. *syringae* strain B728a, which causes brown spot disease in snap bean (Greenberg and Vinatzer 2003). In the well characterised *Pseudomonas syringae* pv. *tomato* DC3000 strain, the insertion of a transposon into the HopAG1 open reading frame (ORF) results in the loss of the C-terminal Nudix hydrolase domain from the effector (Greenberg and Vinatzer 2003, Schechter *et al.*, 2004). Similarly, *Pseudomonas syringae* pv. *avellanae* strain Ve037 has a frameshift mutation which disrupts the HopAG1 effector sequence (O'brien *et al.*, 2012). The loss of HopAG1 in multiple *Pseudomonas syringae* pathovars indicates that the effector is not essential for pathogen virulence. The translocation of HopAG1 into *A. thaliana* leaves was confirmed by fusing the C-terminus of the recognised effector AvrRpt2 to HopAG1 in *P. syringae* pv. *tomato* DC3000 and observing an Rpt2-dependant HR (Vinatzer *et al.*, 2005). HopAG1 is a large effector (716 amino acids) with three predicted domains. At the C-terminus is the Nudix hydrolase domain, at the N-terminus is a predicted ADP-ribosyltransferase domain (Hulin *et al.*, 2023), and the middle domain has a predicted structure similar to proteins that utilise ATPase activity to transfer phosphate to a target (e.g. protein kinases and aminoglycoside phosphotransferases) (Figure 3). The enzymatic activities and roles of the three domains of HopAG1 remain unknown. However, the combination of domains predicted to act on similar substrates suggests HopAG1 may couple multiple enzymatic reactions together, to create small nucleotide/sugar molecules (Figure 3). For example, ADP-ribosyltransferases cleave the N-glycoside bond in NAD⁺, resulting in ADPR. Aminoglycoside phosphotransferases can transfer phosphate to free hydroxyl groups on sugars, from ADPR this may result in ADPR 1-phosphate. The Nudix domain could then hydrolyse the pyrophosphate bond, producing the metabolic activators ribose 1,5-bisphosphate and AMP (Figure 3). While the HopAG1 effector is not essential for the virulence of all *Pseudomonas syringae* pathovars, the intriguing three domain structure is worthy of further investigation.

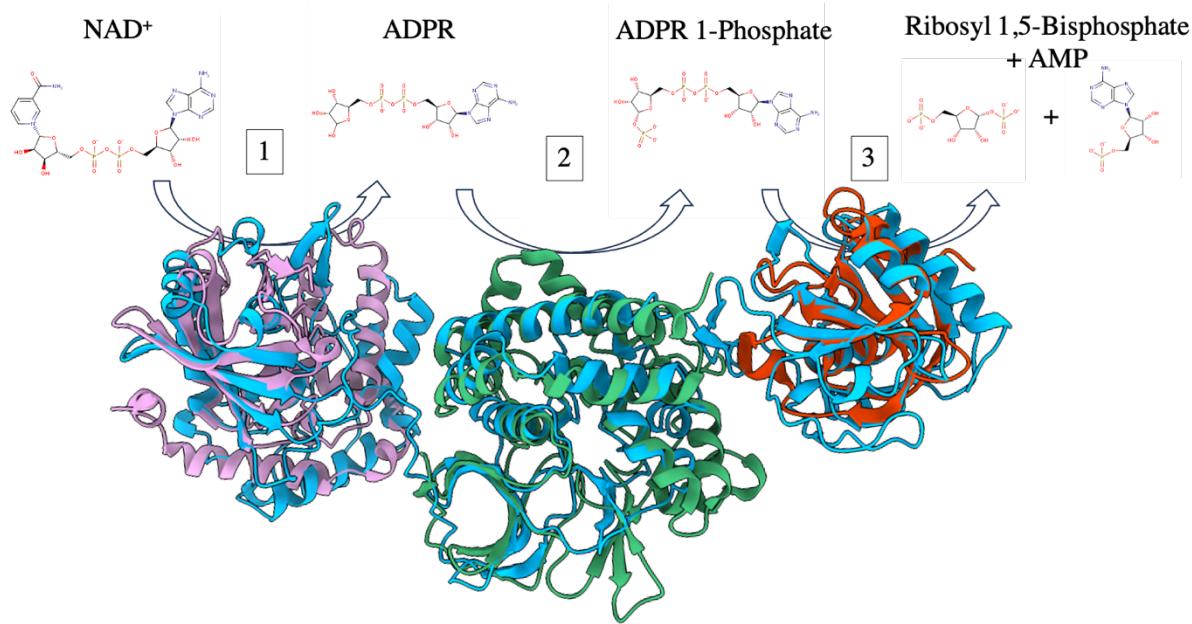


Figure 3: The *Pseudomonas syringae* HopAG1 effector consists of three predicted enzymatic domains. (Top) A hypothetical reaction catalysed by the three predicted enzymatic domains in HopAG1. (1) NAD⁺ could be cleaved by the predicted ADP-ribosyltransferase domain, resulting in ADPR and nicotinamide (not shown). (2) The predicted phosphotransferase domain could transfer a phosphate from ATP (not shown) to ADPR, creating ADPR 1-Phosphate. (3) The predicted Nudix hydrolase domain could use H₂O (not shown) to hydrolyse the pyrophosphate bond in ADPR 1-Phosphate, resulting in Ribosyl 1,5-Bisphosphate and AMP. (Bottom) The predicted structure of *Pseudomonas syringae* pv. *papulans* HopAG1 from the AlphaFold Protein Structure Database (ID: A0A0P9XY22) (cyan) (Jumper *et al.*, 2021, Varadi *et al.*, 2022) aligned with the crystal structures of a *Vibrio splendidus* ADP-ribosyltransferase (PDB ID: 4XZJ) (pink), a *Helicobacter pylori* proinflammatory kinase CTKA (PDB ID: 3AKK) (green), and a *Homo sapiens* Nudix hydrolase MTH1 (PDB ID: 5GHI) (red).

1.3.3 MoNudix

The Nudix effectors predicted in pathogenic fungi are smaller than Avr3b, RipN, and HopAG1, with only slightly more amino acids in the mature effector proteins than that required to achieve the Nudix fold (approximately 130 amino acids are required for the α - β - α sandwich domain architecture). For example, *M. oryzae* has two identical Nudix effector genes (MGG_14344 and MGG_14156, called *MoNudix*) encoding a predicted mature protein of 138 amino acids. *MoNudix* is one of the most highly upregulated effectors during *M. oryzae* infection of rice (Yan *et al.*, 2023), suggesting it may play an important role in promoting pathogen virulence. No substrates have been identified for *MoNudix*, nor is there any experimental evidence indicating *MoNudix* is an active Nudix hydrolase enzyme. However, a potential homologue of *MoNudix*, *CtNudix* from *Colletotrichum lenti*s, elicits a cell-death response when transiently

overexpressed in *N. tabacum* (Bhaduria *et al.*, 2013). When the Nudix motif was deleted from the effector it abolished the cell death phenotype (Bhaduria *et al.*, 2013). However, the deletion of 23 amino acids from the core of the protein likely resulted in major structural changes and therefore it is unclear if the cell death response is dependent on hydrolase activity or a non-enzymatic function of the protein. It is unknown if *CtNudix* possesses hydrolase activity or what substrate it might hydrolyse during infection. Cell-death also required secretion of the effector protein out of the plant cell; this suggests that cell death may be unrelated to the virulence function of the Nudix effector as all Nudix effectors are predicted to act within the plant cell during infection. Likewise, all *A. thaliana* Nudix hydrolases are intracellular proteins (Yoshimura and Shigeoka 2015). More research is required to uncover the roles of *MoNudix*, *CtNudix* and other Nudix effectors from *Colletotrichum* and *Magnaporthe* spp. during the infection process.

1.3.4 AvrM14

In 1965, Harold Flor demonstrated that flax rust isolates with the pathogenicity factors A-M1 and A-M4 show identical segregation on flax cultivars with M1 and M4 resistance (Flor 1965). Over 50 years later, Anderson *et al.*, (2016) demonstrated that flax M1 and M4 resistance proteins both recognise the same flax rust effector protein (named AvrM14), explaining the inseparable genetic linkage between A-M1 and A-M4 observed in Flor's experiments. M1 encodes a TIR-NLR, M4 is yet to be cloned (Lawrence *et al.*, 2010). *M. lini* produces AvrM14 early during the flax infection process (Wu *et al.*, 2019), and other *Melampsora* species encode predicted AvrM14 homologues. AvrM14 has a mature protein size of 146 amino acids and possesses a Nudix box motif (Anderson *et al.*, 2016). The AvrM14 effector has two identified allelic variants, AvrM14-A which is recognised by M1 and M4, and AvrM14-B which is not recognised and enables the infection of M1 and M4 flax cultivars (Anderson *et al.*, 2016). Only six amino acid substitutions distinguish AvrM14-A from AvrM14-B, and both have an identical Nudix box sequence. The recognition of AvrM14-A in M1 and M4 flax when the effector is transiently expressed by agrobacterium-mediated transformation, requires removal of the signal peptide. This demonstrates that AvrM14-A must be retained in the plant cell to enable effector recognition, indicating that AvrM14 is translocated inside flax cells during infection (Anderson *et al.*, 2016). AvrM14 has no identified or predicted substrates.

Table 1: Nudix hydrolase effectors in phytopathogens.

Nudix effector*	Pathogenic species	Mature protein size (amino acids)	Identified <i>in vitro</i> substrates
Avr3b	<i>Phytophthora sojae</i>	297	cNMPs, NADH, ADPR
RipN	<i>Ralstonia solanacearum</i>	473	ADPR, NADH, NAD, FAD, Ap ₄ A
HopAG1	<i>Pseudomonas syringae</i>	716	None
MoNudix	<i>Magnaporthe oryzae</i>	138	None
AvrM14	<i>Melampsora lini</i>	146	None

* Where multiple effectors with greater than 30% protein sequence identity have been identified, only one of the effectors is listed. For example, *CtNudix* is not listed as it has a similar sequence to *MoNudix*.

1.4 Aims of this study

The overall aim of the work presented in this thesis is to characterise the virulence function/s of the Nudix effectors from phytopathogenic fungi, focusing on AvrM14 from *M. lini* and *MoNudix* from *M. oryzae*. I hypothesise that the Nudix effectors from phytopathogenic fungi are enzymes, and that enzymatic activity is important for their virulence function in plants.

To identify molecules hydrolysed by the Nudix effectors, I expressed and purified AvrM14 and *MoNudix* without their signal peptides from *E. coli* to enable characterisation of their enzymatic activity. I also determined crystal structures of both AvrM14 and *MoNudix* proteins to aid in substrate identification and characterisation. I complemented the *in vitro* experimentation with *in planta* analysis, and extended my findings to homologous Nudix effectors in other pathogenic fungal species identified throughout the course of this study.

1.5 References

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Chapter 2 A rust-fungus Nudix hydrolase effector decaps mRNA *in vitro* and interferes with plant immune pathways

To understand the roles of fungal Nudix hydrolase effectors, I sought to characterise AvrM14 from *M. lini*. For the purposes of my thesis, I present this work as a published manuscript. Below is a detailed description of the experimental work performed by myself and others.

Prior to my PhD, Dr Ann-Maree Catanzariti, Dr Simon Williams, and Dr Anna Desai performed experiments to understand the function and recognition of AvrM14. Dr Catanzariti created a series of reciprocal single and double amino acid substitutions in AvrM14-A and AvrM14-B to identify the requirements for recognition by the M1 and M4 resistance proteins. Dr Williams determined the crystal structure of AvrM14-A revealing a Nudix fold, and Dr Desai completed an extensive substrate screening experiment with purified AvrM14-A protein and identified ^{m7}Gp₅G as a substrate of AvrM14-A. The hydrolysis of ^{m7}Gp₅G in the screening experiment suggested that AvrM14 may remove the protective 5' cap from eukaryotic mRNA. During my Honours research year in the Williams lab, I reproduced the results showing hydrolysis of ^{m7}Gp₅G by AvrM14-A and demonstrated that this activity is conserved in AvrM14-B and dependant on a predicted Mg²⁺ co-ordinating glutamate within the Nudix motif. Utilizing the Australian Synchrotron, I collected X-ray diffraction data from crystals of monomeric and homodimeric AvrM14-B proteins to enable structural determination and comparative analysis. I also identified that both AvrM14-A and AvrM14-B could remove the protective 5' cap structure from mRNA *in vitro*.

During my PhD, I refined the previously collected X-ray diffraction data to create accurate models of the crystal structures and deposited these to the protein data bank (PDB) (IDs: 8DP8, 8DP9, and 8DPA). I repeated all mRNA decapping assays with AvrM14-A and AvrM14-B with new preparations of purified protein and different mRNA sequences to ensure the data is robust, reproducible, and to obtain clear images for publication.

Furthermore, I:

1. demonstrate that homodimerisation alters the pyrophosphate bond in the mRNA cap hydrolysed by AvrM14-A and -B,
2. analysed the oligomeric state of AvrM14 when expressed in *N. benthamiana* plants,
3. determined that four additional sequence related Nudix effectors from the *Melampsora* genus retain mRNA decapping activity,

4. generated and analysed RNA-Sequencing datasets to investigate the impact the enzymatic activity of AvrM14 has on flax leaves,
5. identified that the enzymatic activity of AvrM14-A can suppress the HR triggered by the M1 resistance protein in flax,
6. repeated experiments initially completed by Dr Catanzariti demonstrating that the Nudix hydrolase activity of AvrM14 suppresses flg-22 and chitin triggered ROS production in *N. benthamiana*.

For publication, I assembled all data collected on the function and recognition of AvrM14. I wrote the initial draft of the manuscript and created all the figures. The draft was subsequently edited and approved by all authors. This chapter includes the main text and supporting information as published in *New Phytologist* and available online at <https://doi.org/10.1111/nph.18727>. The statement of contribution details my pivotal role in this work.



A rust-fungus Nudix hydrolase effector decaps mRNA *in vitro* and interferes with plant immune pathways

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Summary

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- To infect plants, pathogenic fungi secrete small proteins called effectors. Here, we describe the catalytic activity and potential virulence function of the Nudix hydrolase effector AvrM14 from the flax rust fungus (*Melampsora lini*).
- We completed extensive *in vitro* assays to characterise the enzymatic activity of the AvrM14 effector. Additionally, we used *in planta* transient expression of wild-type and catalytically dead AvrM14 versions followed by biochemical assays, phenotypic analysis and RNA sequencing to unravel how the catalytic activity of AvrM14 impacts plant immunity.
- AvrM14 is an extremely selective enzyme capable of removing the protective 5' cap from mRNA transcripts *in vitro*. Homodimerisation of AvrM14 promoted biologically relevant mRNA cap cleavage *in vitro* and this activity was conserved in related effectors from other *Melampsora* spp. *In planta* expression of wild-type AvrM14, but not the catalytically dead version, suppressed immune-related reactive oxygen species production, altered the abundance of some circadian-rhythm-associated mRNA transcripts and reduced the hypersensitive cell-death response triggered by the flax disease resistance protein M1.
- To date, the decapping of host mRNA as a virulence strategy has not been described beyond viruses. Our results indicate that some fungal pathogens produce Nudix hydrolase effectors with *in vitro* mRNA-decapping activity capable of interfering with plant immunity.

Introduction

Infectious diseases represent a significant threat to global crop production. To facilitate and maintain infection, plant pathogens secrete an array of proteins (termed effectors; Lo Presti *et al.*, 2015). Effectors are either delivered to the apoplast or translocated inside host cells, to modulate cellular processes. Plants have evolved resistance genes (*R*-genes) to defend against pathogen infection. Resistance genes encode effector-detecting resistance proteins (R-proteins) capable of inducing a strong immune response, known as effector-triggered immunity (ETI; Jones & Dangl, 2006; Dodds & Rathjen, 2010). The activation of ETI typically prevents pathogen growth, and recognised effectors are therefore referred to as avirulence (Avr) proteins. The ability of effectors to promote infection while avoiding recognition is crucial for successful infection. Consequently, understanding the pathogenicity function of effectors and how they are

perceived by R-proteins remains a major focus in the study of plant–pathogen interactions.

Nucleoside diphosphate linked to moiety-X (Nudix) hydrolase domains have been predicted in effectors from multiple fungal, oomycete and bacterial plant pathogens (Dong & Wang, 2016). The putative Nudix hydrolase effectors were identified based on similarities with the Nudix-box consensus sequence (GX₃EX₇-REUXEEXGU, where *U* is usually a hydrophobic residue and *X* is any amino acid). Nudix hydrolases typically hydrolyse nucleoside diphosphates bound to an additional moiety and substrate specificity is generally controlled by residues outside the Nudix box (McLennan, 2006; Srouji *et al.*, 2017). Well-characterised substrates of Nudix hydrolases include nucleoside triphosphates (Sakumi *et al.*, 1993; Ito *et al.*, 2005; Jemth *et al.*, 2018), adenosine diphosphate ribose (ADPR) (O'Handley *et al.*, 1998; Gabelli *et al.*, 2002; Kang *et al.*, 2003; Perraud *et al.*, 2003), diadenosine polyphosphates (ApnAs; Bessman *et al.*, 2001; Leslie *et al.*, 2002; Iwai *et al.*, 2004) and the 5' cap of mRNA transcripts (Van Dijk

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et al., 2002; Wang et al., 2002; Song et al., 2013). Nudix hydrolases are ubiquitous in prokaryotic and eukaryotic organisms and are involved in diverse physiological processes and cellular homeostasis (McLennan, 2006).

The first Nudix hydrolase effector to be experimentally validated was Avr3b from the oomycete *Phytophthora sojae*, a destructive pathogen of soybean. Transient expression of Avr3b in *Nicotiana benthamiana* increased susceptibility to *Phytophthora capsica* and *Phytophthora parasitica* infection, diminished reactive oxygen species (ROS) accumulation around invasion sites, and reduced plant cell death during infection (Dong et al., 2011). In soybean, the expression of Avr3b suppressed cell death triggered by the recognition of Avr1b (Dong et al., 2011). Avr3b requires a plant cyclophilin for enzymatic activation, and both the cyclophilin and the Nudix box are required for the identified Avr3b virulence activities (Dong et al., 2011; Kong et al., 2015). Avr3b hydrolyses multiple nucleoside diphosphate compounds *in vitro* (Dong et al., 2011). Recently, Yu et al. (2022) demonstrated that Avr3b also has phosphodiesterase activity against 2',3'-cAMP/GMP and suggest that this enzymatic activity is a virulence function of the effector (Yu et al., 2022). The promiscuous *in vitro* enzymatic activity of Avr3b is common among Nudix hydrolases (McLennan, 2013) and complicates the identification of the true substrates of the effector during infection. RipN, a Nudix hydrolase effector from the bacterial pathogen *Ralstonia solanacearum*, also hydrolyses a variety of nucleoside diphosphate substrates *in vitro* (Sun et al., 2019). The preferred *in vitro* substrates of RipN are NADH and ADPR; however, overexpression of RipN in *Arabidopsis thaliana* did not result in significant changes to ADPR, NAD⁺ or NADH levels (Sun et al., 2019). CtNUDIX, a putative Nudix hydrolase effector from the hemibiotrophic fungus *Colletotrichum truncatum*, elicits a cell-death response when transiently expressed in *Nicotiana tabacum* (Bhaduria et al., 2013). However, it is unknown whether CtNUDIX possesses hydrolase activity and what it targets during infection. Putative Nudix hydrolase effectors have also been identified in biotrophic fungal pathogens. The flax rust-fungus *Melampsora lini* expresses AvrM14 early in infection of flax. AvrM14 encodes a predicted Nudix hydrolase effector (Anderson et al., 2016; Wu et al., 2019). The AvrM14 effector is recognised by the flax immune receptors M1 and M4, with previous research on AvrM14 immune recognition indicating that the effector is translocated inside flax cells during infection (Anderson et al., 2016). Here, we report the structure, biochemical activity and likely virulence function of the AvrM14 protein.

Materials and Methods

Cloning

AvrM14-A and AvrM14-B, without the predicted signal sequence (residues 22–166), were PCR-amplified from *Melampsora lini* cDNA (primers listed in Supporting Information Table S1) to facilitate respective downstream cloning processes.

AvrM14 mutants were generated using the Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) and 5' phosphorylated primers (Table S1).

For transient expression of AvrM14 *in planta*, T-DNA plasmids were generated using Gateway cloning. The constructs were initially cloned into the pENTR/D-TOPO entry vector and then into the pEarleyGate (pEG) destination vectors pEG102 or pEG104 (Earley et al., 2006). All constructs were cloned with 3' stop codons after the coding sequence, resulting in the expression of untagged (pEG102) or N-terminal YFP-tagged (pEG104) proteins *in planta*. The M1 sequence was cloned into the pTN35S plant expression vector (Dodds et al., 2004).

For recombinant protein production in *Escherichia coli*, the AvrM14-A, AvrM14-B, AvrM14-A^{E82Q} and AvrM14-B^{E82Q} sequences were cloned into the pMCSG7 vector using ligation-independent cloning (Stols et al., 2002). The resulting plasmids encoded an N-terminal hexahistidine (6×His) tag, followed by a tobacco etch virus protease cleavage site allowing 6×His tag removal. AvrM14 homologues from other *Melampsora* spp. were ordered as *E. coli* codon-optimised double-stranded DNA fragments without the predicted signal sequences from Integrated DNA Technologies Inc. (IDT®, Coralville, IA, USA). The DNA fragments were cloned into a modified pOPIN vector (Bentham et al., 2021), resulting in an N-terminal 6×His tagged construct with a 3C protease site in-between the 6×His tag and the coding sequence for each homologue. All protein sequences following purification and tag-cleavage are listed in Table S1.

Protein production

Recombinant HsNudt16 protein was purchased from Abcam (Cambridge, UK). All other proteins were expressed in *E. coli* BL21 (DE3) cells and purified using nickel metal affinity chromatography, followed by size-exclusion chromatography. For full details of protein production, see Methods S1.

Crystallisation and structure determination

Initial crystallisation screening with purified AvrM14-A and AvrM14-B monomeric proteins was conducted using a Mosquito robot (STP LabTech, Melbourn, UK) in a 96-well plate format using sparse matrix screens. The hanging drop vapour-diffusion method of crystallisation was used and drops consisting of 100 nl 10 mg ml⁻¹ protein solution and 100 nl reservoir solution were equilibrated against 100 µl reservoir solution. The final optimised condition for monomeric AvrM14-A (10 mg ml⁻¹) was 2.4 M sodium malonate pH 6.0 and 2% glycerol. For monomeric and homodimeric AvrM14-B, the condition utilised for crystallisation was 1.8 M (NH₄)₂SO₄, 100 mM sodium acetate (pH 4.33) and 100 mM MgCl₂.

Before X-ray data collection, AvrM14-A crystals were transferred to 2.4 M sodium malonate pH 6.0 and 10% glycerol, before flash-cooling in liquid nitrogen. To enable phasing, some AvrM14-A crystals were soaked in a solution of 2.4 M sodium malonate pH 6.0, 10% glycerol and 1 M NaBr. AvrM14-B

crystals were transferred to the same condition used for crystallisation supplemented with 20% glycerol, before flash-cooling in liquid nitrogen.

Single-wavelength anomalous diffraction (SAD) and native datasets were collected on the MX1 and MX2 beamlines at the Australian Synchrotron (Table S2; Cowieson *et al.*, 2015; Aragão *et al.*, 2018). The datasets were processed in XDS (Kabsch, 2010) and scaled using AIMLESS in the CCP4 suite (Evans & Murshudov, 2013). For SAD phasing of AvrM14-A, the CRANK2 pipeline (Skubák & Pannu, 2013) was used in the CCP4 suite. The best chain from the autobuilt model was then used as a search model to solve the structure of a native dataset using maximum-likelihood molecular replacement (MR) with Phaser in Phenix (Liebschner *et al.*, 2019).

The crystal structure of monomeric and homodimeric AvrM14-B was determined using maximum-likelihood MR with Phaser in Phenix (Liebschner *et al.*, 2019). The MR search model was chain A of the AvrM14-A crystal structure. For all native datasets, automated model building and initial refinement were completed using either Phenix AutoBuild (Terwilliger *et al.*, 2008) or ARP/wARP (Langer *et al.*, 2008). Subsequent model building was carried out manually in Coot (Casañal *et al.*, 2020) in-between rounds of automated refinement using Phenix refine (Afonine *et al.*, 2012). MolProbity was used for validation of the final models (Williams *et al.*, 2018). Analysis of the final structures was performed with Coot (Casañal *et al.*, 2020), PyMOL, APBS (Jurrus *et al.*, 2018) and ESPRIPT v.3.0 (Robert & Gouet, 2014).

Map coordinates and structure files have been deposited in the Protein Data Bank (AvrM14-A Monomer: 8DP8, AvrM14-B Monomer: 8DP9, AvrM14-B Homodimer: 8DPA).

Nudix hydrolase substrate screening assays

To assess phosphohydrolase activity against ADPR, NADH, Ap₄A, FAD and NAD⁺, 6 μM of recombinant protein was incubated with 2 mM substrate in a reaction buffer containing 50 mM Tris-HCl pH 8.5, 5 mM MgCl₂, 1 mM DTT and 40 U ml⁻¹ of alkaline phosphatase at 37°C for 30 min. To detect inorganic phosphate produced from the reactions, a phosphomolybdate method was used (Ames, 1966). Sulphuric acid was added to a final concentration of 500 mM; ammonium molybdate was added to a final concentration of 2 mM and ascorbic acid to a final concentration of 60 mM; the solution was then incubated at 45°C for 10 min, and the absorbance at 820 nm was recorded.

A kinetic screening method with a phosphate sensing fluorophore (Nguyen *et al.*, 2016) was used to measure phosphohydrolase activity against 69 unique nucleoside diphosphates (substrates and their groups are listed in Table S3). In short, the standard reaction contained 10 mM Tris-HCl pH 7.6, 1 mM MgCl₂, 5 μM phosphate sensor (Thermo Fisher Scientific), 0.05 U ml⁻¹ of yeast pyrophosphatase (PPase) when pyrophosphate was a predicted product or 1 U ml⁻¹ of alkaline phosphatase (APase) when a nucleoside monophosphate was a predicted product. Each substrate concentration was 5 μM in both grouped and ungrouped reactions. AvrM14-A recombinant protein concentration was 50 nM. The

mixtures were incubated at 37°C and monitored continuously for 30 min on a Tecan GENios Microplate Reader with the following parameters: λ-excitation = 425 nm, λ-emission = 465 nm, gain 60, 100 cycles, 37°C.

The subsequent kinetic screen to assess AvrM14-A, AvrM14-B, AvrM14-A^{E82Q} and AvrM14-B^{E82Q} hydrolase activity against ^{m7}Gp₅G (Jena Bioscience, Jena, Germany) was completed as above, with some modifications to optimise activity. The reaction mixture contained 10 mM Tris-HCl (pH 7.6), 2 mM MnCl₂, 1 U ml⁻¹ alkaline phosphatase, 5 μM ^{m7}Gp₅G, 2.5 μM phosphate sensor (Thermo Fisher Scientific) and 100 nM purified recombinant protein in a total volume of 100 μl. Fluorescence measurements (excitation at 425 nm and emission at 465 nm) were recorded every 10 s for 30 min using a Tecan Infinite® M1000 (Tecan, Männedorf, Switzerland) plate reader at room temperature.

RNA synthesis, purification, capping and decapping

A 231-nt synthetic RNA sequence (sequence in Table S1) was synthesised using the HiScribe™ T7 High Yield RNA Synthesis Kit (NEB, Ipswich, MA, USA) following the recommended protocol. The RNA was purified using RNAClean XP beads (Beckman Coulter, Brea, CA, USA). Purified RNA was capped using the Vaccinia Capping System (NEB) with GTP spiked with (α-³²P)-GTP (PerkinElmer, Waltham, MA, USA). To purify the capped RNA, the Monarch® RNA Cleanup Kit (NEB) was used, and two purifications were completed per capping reaction to ensure near-complete removal of (α-³²P)-GTP.

RNA-decapping assays were completed as described previously, with some minor modifications (Song *et al.*, 2013). In brief, 2 μM recombinant protein was incubated with capped RNA in a reaction buffer with 10 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM MnCl₂, 1 mM DTT and 40 U ml⁻¹ RNase Inhibitor (Merck, Rahway, NJ, USA) for 30 min at 37°C. The reaction products were separated using PEI-cellulose TLC plates (Merck) with 0.45 M (NH₄)₂SO₄ as the liquid phase. Dried plates were placed into a cassette with a K-screen (Bio-Rad) and left for 14–18 h before imaging using a PharosFX™ scanner (Bio-Rad). To identify the reaction products, known standards of ^{m7}GMP (Jena Bioscience), ^{m7}GDP (Jena Bioscience), GMP (Merck) and GDP (Merck) were separated using the same TLC conditions and imaged using UV shadowing.

Plant materials and growth conditions

Nicotiana benthamiana and *N. tabacum* L. plants were grown in soil in a controlled environment at 25°C with a 16-h day length. Flax (*Linum usitatissimum* L.) plants were grown in soil in glasshouses with natural light at a constant temperature of 24°C. Infiltration experiments were completed on 4–5-wk-old *N. benthamiana*, c. 7-wk-old *N. tabacum* and c. 3-wk-old *L. usitatissimum*.

Agroinfiltrations

Agroinfiltrations were completed as described previously (Catanarziti *et al.*, 2015). In brief, *Agrobacterium* strain GV3101

(pMP90) containing the pEG102, pEG104 or pTN35S DNA constructs (see 'Cloning' in the Materials and Methods section) were suspended in infiltration buffer (10 mM MES pH 5.6, 10 mM MgCl₂ and 200 μM acetosyringone) to a final optical density at 600 nm of 1.0. All cultures were incubated in the dark at room temperature for 2 h before syringe-infiltration into either *N. benthamiana*, *N. tabacum* or *L. usitatissimum* leaves. Infiltrated plants were kept in the same growing conditions as before infiltration.

ROS burst assays

Measurement of ROS was completed as described previously with some minor modifications (Heese *et al.*, 2007). In brief, *N. benthamiana* leaf discs (4 mm diameter) were floated on water overnight in a 96-well plate. The water was replaced with an elicitor solution (100 μM luminol, 10 μg ml⁻¹ horseradish peroxidase and 100 nM flg-22 or 5 μg ml⁻¹ chitin), and luminescence was measured over time using a Tecan Infinite® M1000 Pro (Tecan) plate reader at room temperature.

Immunoblot analysis

Rabbit polyclonal antibodies against recombinant AvrM14-A protein were produced and affinity purified by the Walter & Eliza Hall Institute of Medical Research (WEHI, Melbourne, Vic., Australia). Protein extraction from plant tissue for Western blotting was performed as described previously (108). Blots were probed with either rabbit anti-AvrM14 (pEG102 constructs) or mouse anti-GFP (pEG104 constructs; Roche) and detected by goat anti-rabbit-HRP (Merck) or goat anti-mouse-HRP (Cytiva, Marlborough, MA, USA). Clarity™ Western ECL substrate (Bio-Rad) was added to the immunoblots and chemiluminescence detected using a ChemiDoc imager (Bio-Rad).

SEC-MALS analysis

SEC-MALS was performed as described previously (Casey *et al.*, 2016). In brief, the purified protein samples were loaded onto a Superdex 75 Increase 10/300 size-exclusion column (Cytiva) pre-equilibrated in buffer (10 mM HEPES pH 7.5, 150 mM NaCl), connected to a Dawn Heleos II 18-angle light-scattering detector and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The molecular masses of the proteins were calculated using ASTRA 6.1 software (Wyatt Technology).

RNA extractions, sequencing and analysis

Twenty flax leaves from a single plant (per replicate, with a total of four replicates for both the AvrM14-A and AvrM14-A^{E82Q} treatments and three replicates for the vector-only treatment) were collected 3 d post-infiltration with either the pEG102 AvrM14-A, pEG102 AvrM14-A^{E82Q} or empty vector (pEG102) GV3101 *Agrobacterium* constructs. Each replicate (20 leaves) was placed into a plastic tube with stainless steel beads and frozen in

liquid nitrogen. Plant tissue was lysed using the TissueLyser II (Qiagen). TRIzol™ reagent and the PureLink™ RNA Mini Kit (Thermo Fisher Scientific) were used for RNA purification from the lysed sample following the manufacturer's protocol.

Whole transcriptome RNA sequencing was conducted by Novogene (Beijing, China) using the Ribo-Zero Magnetic Kit (Illumina, San Diego, CA, USA) for rRNA depletion, random hexamers for cDNA synthesis, the NEBNext® Ultra™ Directional RNA Library Prep Kit (NEB) for library preparation and the Illumina Novaseq 6000 platform for sequencing. Raw sequencing data is available from the NCBI (GEO: GSE207874, SRA: SRX16112399, SRX16112400, SRX16112401, SRX16112402, SRX16112403, SRX16112404, SRX16112405, SRX16112406, SRX16112384, SRX16112385 and SRX16112386). For full details of RNA-Seq data analysis, see Methods S1.

Results

AvrM14 is an active Nudix hydrolase capable of interfering with plant immune responses

The AvrM14 effector from flax rust contains 146 amino acids as a mature protein that includes a Nudix-box consensus sequence (Anderson *et al.*, 2016). Two allelic variants of AvrM14 have been identified (AvrM14-A and AvrM14-B). AvrM14-A is recognised by flax R-proteins M1 and M4, while AvrM14-B possesses six amino acid polymorphisms and has escaped recognition (Anderson *et al.*, 2016). To understand the virulence function(s) of AvrM14 and how the AvrM14-B effector escapes recognition by M1 and M4, we determined the crystal structures of AvrM14-A and AvrM14-B at a resolution of 2.3 and 1.8 Å, respectively (see Table S2 for data collection and refinement statistics; the higher-resolution structure of AvrM14-B will be used for structure analyses unless indicated otherwise). The structures revealed that AvrM14 proteins display a characteristic Nudix-fold architecture, consisting of a β-grasp fold surrounding an α-helix (Fig. 1a; Lin *et al.*, 1997; Gabelli *et al.*, 2001). In typical Nudix hydrolases, the Nudix box folds into a loop-α-helix-loop structure, but for AvrM14, a 3₁₀-helix is present before the α-helix (Figs 1a, S1a). The 3₁₀-helix appears to compensate for the double amino acid insertion in the AvrM14 Nudix box (Anderson *et al.*, 2016), allowing the protein to maintain a salt bridge between a conserved Nudix-box glutamate (E71) and arginine (R81) found in canonical Nudix hydrolase structures (Fig. S1a; Gabelli *et al.*, 2001; Xu *et al.*, 2004). Nudix hydrolases require divalent metal ions for catalysis (Mildvan *et al.*, 2005; McLennan, 2006). While Mg²⁺ cations were included in our crystallisation solution for AvrM14-B, we did not observe clear density to suggest metal ion binding in the structures. Despite this, two of the three Nudix-box glutamate residues typically required for divalent metal binding (E85 and E86) are positioned in a similar location to previously determined metal-bound Nudix hydrolase structures (Fig. S1c; Gabelli *et al.*, 2002). The third Nudix-box glutamate, also often implicated in metal binding (E82), has dual conformations in our crystal structures. In one conformation, the side chain is buried under a β-sheet, whereas in the other, it is solvent-exposed (Fig. S1c). Based on previously determined metal-bound

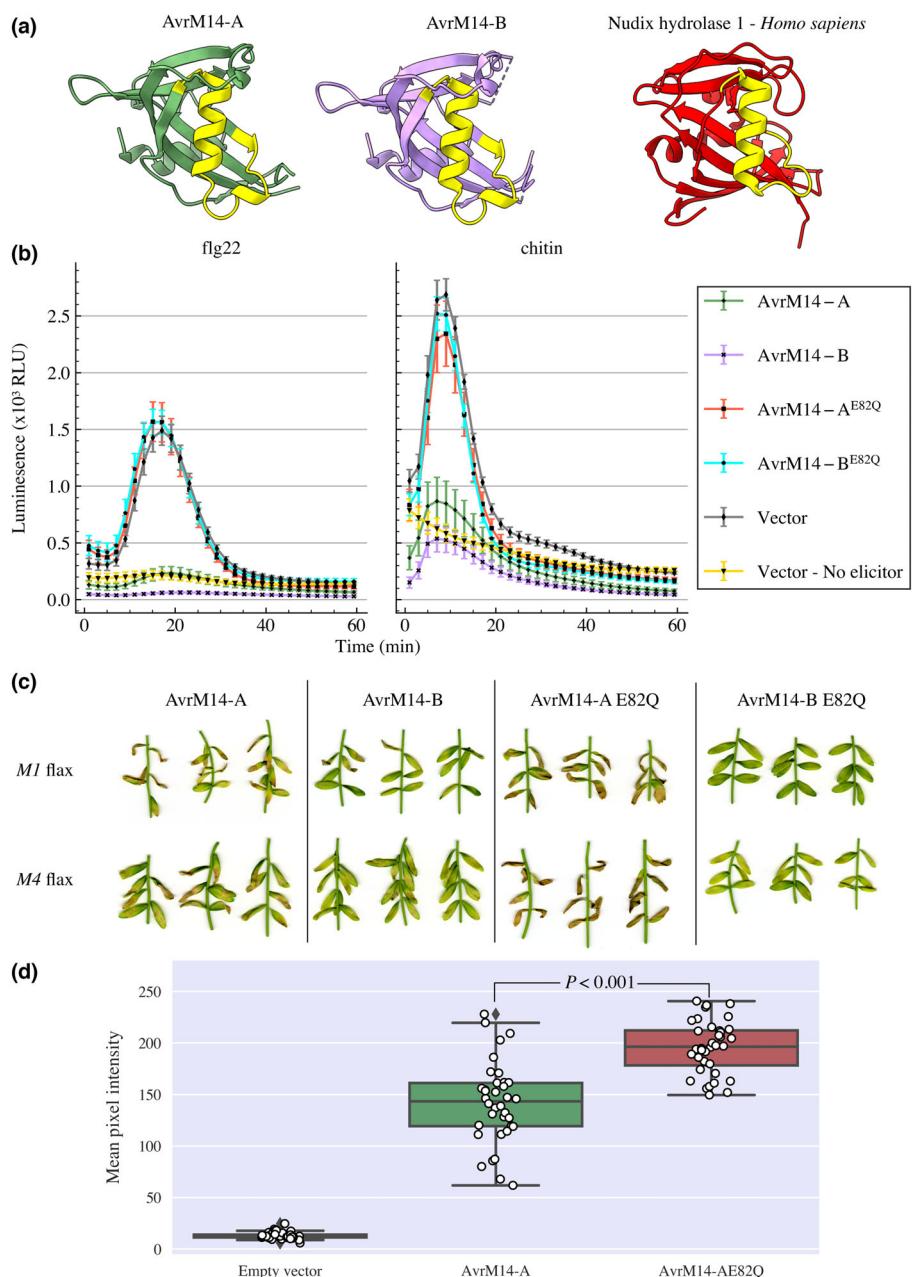


Fig. 1 Enzymatic activity of AvrM14, a Nudix hydrolase, suppresses reactive oxygen species (ROS) production, triggered by flg22 or chitin, and the hypersensitive response, triggered by the M1 resistance protein. (a) Ribbon diagrams of the AvrM14-A and AvrM14-B crystal structures, (PDB ID: 8DP8 and 8DP9) showing they adopt a Nudix fold. The Nudix-box region that typically folds into a loop-α-helix-loop is coloured yellow. For comparison to a prototypical Nudix hydrolase, the structure of Nudix hydrolase 1 (MTH1) from *Homo sapiens* is shown on the right (PDB ID: 5GHI) in analogous orientation (Waz et al., 2017). (b) *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium* (GV3101) harbouring either an empty vector (pEG104) or pEG104 constructs encoding AvrM14-A, AvrM14-B, AvrM14-AE82Q or AvrM14-BE82Q proteins with YFP N-terminal tags expressed under the 35 S promoter from the cauliflower mosaic virus. At 4 d post-infiltration (dpi), leaf tissue was exposed to 100 nM flg-22 or 1 µg ml⁻¹ chitin (except in the no-elicitor treatment) and ROS production was recorded as relative luminescence units over time. Results are means ± SEs (n = 12). (c) Bison × M1 (Williston Brown) or M4 (Victory 'A') flax lines were agroinfiltrated to transiently express either wild-type or mutant AvrM14 proteins (AvrM14-A, AvrM14-B, AvrM14-AE82Q and AvrM14-BE82Q). Multiple leaves from three independent plants were infiltrated with each construct. The agroinfiltrated leaves were photographed 7 dpi. (d) Bison × M1 (Williston Brown) flax leaves were agroinfiltrated with an empty vector or with constructs to transiently express either wild-type or mutant AvrM14-A proteins (AvrM14-A and AvrM14-AE82Q). At 6 dpi, all infiltrated leaves were collected and the cell-death response for each leaf was assessed by pixel intensity analysis using IMAGEJ after imaging the leaves with a Chemidoc MP imager. There were 36 replicates for each treatment, as indicated by the white dots on top overlaying the boxplots. In the boxplots the horizontal line in the middle of the box represents the median value, the box represents the interquartile range (IQR), the whiskers extend to 1.5 × IQR, and the grey diamonds indicate outlying values (defined as <Q1 - 1.5 × IQR or >Q3 + 1.5 × IQR). To determine whether there was a significant difference between the AvrM14-A and AvrM14-AE82Q treatments, an unpaired t-test was completed and the two-tailed P-value is displayed.

Nudix hydrolase structures, we propose that metal ion binding would lock E82 in the solvent-exposed conformation (Gabelli *et al.*, 2002). Overall, the Nudix fold and positioning of the putative metal-binding glutamates suggest that the AvrM14 effectors are enzymatically active Nudix hydrolases.

The RipN and Avr3b effectors both interfere with plant immunity in a Nudix-box-dependent manner, when expressed in *A. thaliana* and *N. benthamiana*, respectively (Dong *et al.*, 2011; Sun *et al.*, 2019). To determine whether AvrM14 can use Nudix hydrolase activity to interfere with plant immunity, we used *Agrobacterium*-mediated transformation in *N. benthamiana* to transiently express AvrM14-A, AvrM14-B, and both proteins with glutamate 82 mutated to glutamine (AvrM14-A^{E82Q} and AvrM14-B^{E82Q}) as YFP fusions and assessed their effect on plant immune responses. Numerous studies have demonstrated this equivalent mutation drastically reduces the catalytic activity of Nudix hydrolases, without altering protein structure or stability (Lin *et al.*, 1996; Perraud *et al.*, 2003; Mildvan *et al.*, 2005; Parrish *et al.*, 2007; Gunawardana *et al.*, 2008; Höfer *et al.*, 2016). Our results demonstrate that the expression of AvrM14-A and AvrM14-B, but not the E82Q mutants, inhibits the production of ROS, following exposure of *N. benthamiana* leaf tissue to either flg-22 or chitin (Fig. 1b). Both AvrM14 and E82Q mutant proteins were expressed and accumulate to similar levels in agroinfiltrated plant tissue (Fig. S2).

As both AvrM14-A and AvrM14-B suppress ROS production, and AvrM14-B is not recognised by the flax M1 and M4 R-proteins, we hypothesised that hydrolase activity would not be required for the recognition of AvrM14-A by M1 and M4. In support of this hypothesis, transient expression of AvrM14-A and AvrM14-A^{E82Q}, but not AvrM14-B or AvrM14-B^{E82Q}, triggered cell-death responses in near-isogenic lines of the flax variety Bison containing either *M1* (*M1* flax) or *M4* (*M4* flax) (Fig. 1c). *M1* and *M4* were introgressed into the Bison genetic background by 12 backcrosses, see Islam & Mayo (1990).

Analogous results were observed in tobacco when the effector was transiently co-expressing with *M1* (Fig. S3). Interestingly, AvrM14-A^{E82Q} expression appeared to result in a stronger cell-death response when compared to the wild-type protein in both *M1* and *M4* flax (Fig. 1c). We confirmed this result by expressing AvrM14-A and AvrM14-A^{E82Q} in a total of 36 *M1* flax leaves per treatment and the HR cell-death response was assessed by determining the mean signal intensity of each infiltrated area following green-light imaging of the leaves (Fig. 1d). The results demonstrate that AvrM14-A^{E82Q} expression results in a greater cell-death response than AvrM14-A (Fig. 1d). Our results indicate that while the integrity of the Nudix box is dispensable for M1 and M4 detection, the AvrM14-A E82Q mutation does influence the severity of the M1 activated immune response. Collectively, these data suggest that AvrM14 can suppress both PTI and ETI pathways, via an unknown Nudix hydrolase activity.

RNA cap analogues and capped RNA transcripts are selectively hydrolysed by AvrM14

To determine the compound(s) hydrolysed by AvrM14, we conducted a comprehensive substrate search, utilising a sensitive

fluorometric assay (Xu *et al.*, 2013), which tested AvrM14-A protein against 69 unique nucleoside diphosphate compounds (listed in Table S3). AvrM14-A only exhibited hydrolysis activity with a single substrate, ^{m7}Gp₅G (P1-(5'-7-methyl-guanosyl)-P5-(5'-guanosyl)-pentaphosphate). AvrM14-A did not hydrolyse diguanosine pentaphosphate (Gp₅G), indicating a preference for the methyl group.

To determine whether substrate hydrolysis was dependent on the Nudix box present in AvrM14-A and conserved in AvrM14-B, we assessed the hydrolytic activity of recombinant AvrM14-A, AvrM14-B, AvrM14-A^{E82Q} and AvrM14-B^{E82Q} against ^{m7}Gp₅G and monitored fluorescence over time (Fig. 2a). AvrM14-A and AvrM14-B hydrolysed ^{m7}Gp₅G, whereas the catalytic site mutants did not (Fig. 2a). ^{m7}Gp₅G is not found in nature; however, 7-methyl guanosine (^{m7}G) is a unique molecular structure present on the 5' cap of eukaryotic RNA transcribed by RNA polymerase II (Müller-McNicoll & Neugebauer, 2014); ^{m7}Gp₅G is used as an mRNA cap analogue in assays. Therefore, the results from the substrate screening experiments suggest that AvrM14 may function as an mRNA-decapping enzyme.

To determine whether the AvrM14 proteins hydrolyse ^{m7}G-capped RNA, we conducted a radiolabelled assay utilising a known decapping enzyme, *Homo sapiens* Nudt16 (HsNudt16) (Song *et al.*, 2010; Li *et al.*, 2011), as a positive control. AvrM14-A and AvrM14-B both efficiently decapped the labelled mRNA substrate, whereas the active site mutants (AvrM14-A^{E82Q} and AvrM14-B^{E82Q}) did not (Fig. 2b). These data demonstrate that AvrM14 is an active Nudix hydrolase with specificity for capped mRNA *in vitro*.

AvrM14 homodimerisation via domain swapping optimises RNA decapping

The AvrM14-A and AvrM14-B crystal structures display a common crystal packing interface despite different crystallisation conditions and crystallographic space groups (Table S2; Fig. S4c). Interestingly, the position of the interface, with respect to the Nudix-box helix, is similar to previously identified homodimeric RNA-decapping enzymes HsNudt16 and X29 (a Nudt16 ortholog from *Xenopus laevis*; Scarsdale *et al.*, 2006; Trésaugues *et al.*, 2015; Fig. S5). We aimed to determine whether homodimerisation of AvrM14 is important for mRNA decapping. Based on SEC (size-exclusion chromatography) chromatograms, we show that both recombinant AvrM14-A and AvrM14-B proteins exist in two stable and distinct oligomeric states that can be purified to near homogeneity (Fig. S4a,b). To determine the molecular mass of each oligomeric state more accurately, we utilised SEC coupled to a multi-angle light-scattering detector (SEC-MALS; Fig. 3). Our SEC-MALS data demonstrate that, in solution, recombinant AvrM14-B forms stable monomers and homodimers, which can be readily separated and purified (Fig. 3a).

The initial crystallisation and activity assays reported above were completed with monomeric AvrM14 proteins, which are the predominant forms after purification from *E. coli* (Fig. S4a). The fact that previous research indicates that HsNudt16 and X29 Nudix hydrolase mRNA-decapping enzymes act as homodimers,

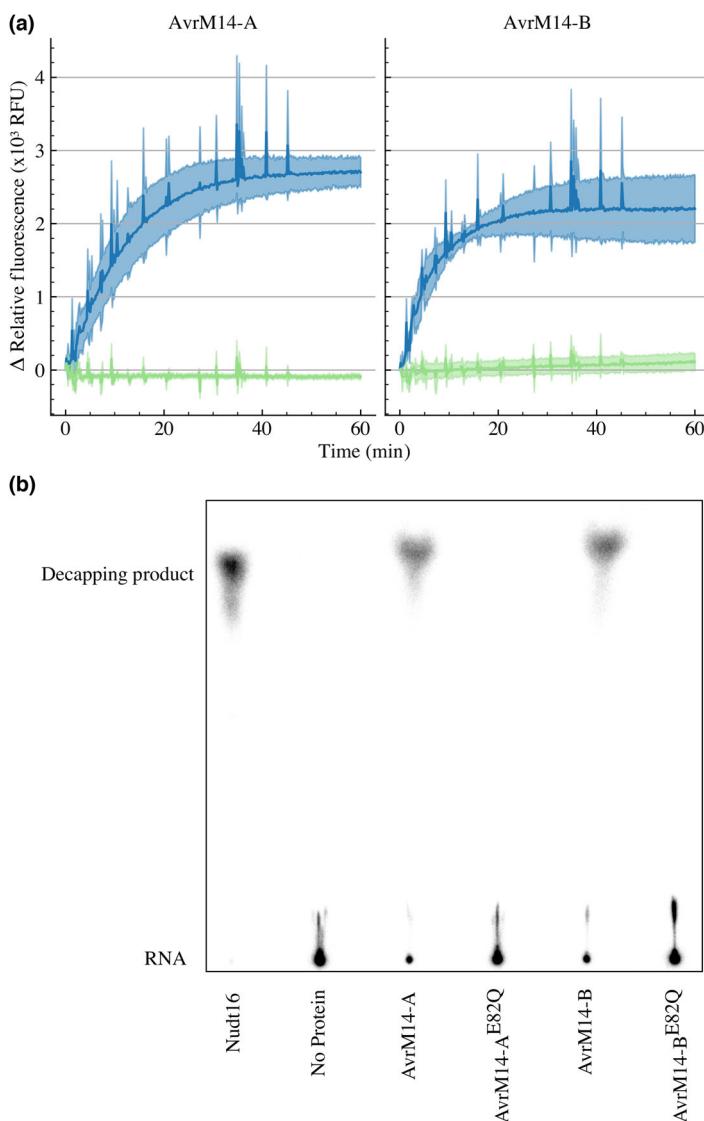


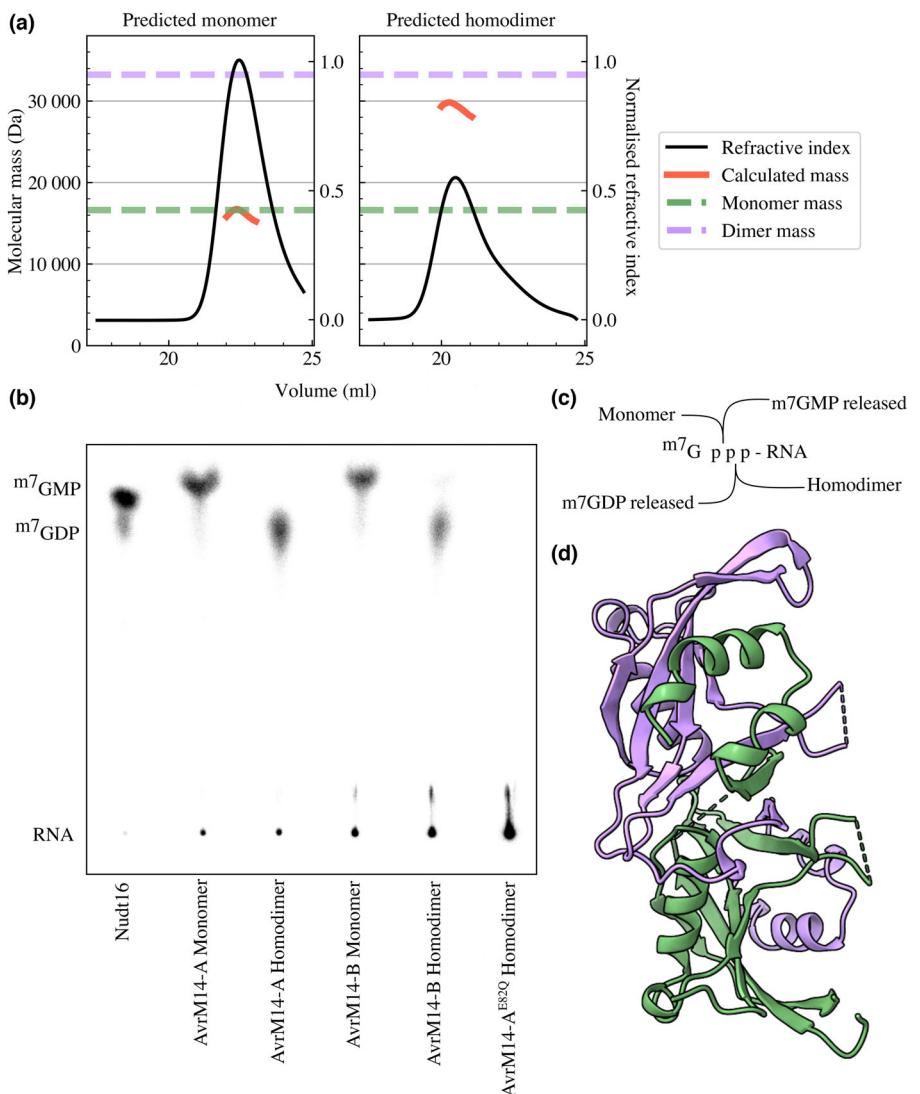
Fig. 2 AvrM14-A and AvrM14-B hydrolyse RNA cap analogues and capped mRNA *in vitro*. (a) Recombinant AvrM14-A (left) and AvrM14-B (right) (data in blue) and the corresponding mutant AvrM14^{E82Q} proteins (green) were incubated with the mRNA cap analogue m^7Gp_5G . Hydrolysis activity was measured over 60 min using a phosphate-binding fluorophore. Results are means (solid line) \pm SDs (shaded area), where $n = 3$. (b) Recombinant proteins (HsNudt16, AvrM14-A, AvrM14-A^{E82Q}, AvrM14-B and AvrM14-B^{E82Q}) were incubated with $m^7Gp^{32}pp$ -RNA and the reaction products analysed by thin-layer chromatography (TLC). Capped RNA remains at the origin of the TLC plate, whereas decapping products (m^7GDP and/or m^7GMP) migrate up the plate.

prompted us to further characterise the homodimeric form of AvrM14. To determine whether homodimerisation alters substrate specificity, we performed substrate screening with five common Nudix hydrolase substrates (Fig. S6). Consistent with the monomeric forms of the protein, homodimeric AvrM14-A and AvrM14-B did not hydrolyse any of the substrates tested (Fig. S6). To determine whether homodimeric AvrM14 proteins could decap RNA, we performed activity assays with m^7G -capped RNA. Both AvrM14-A and -B homodimers hydrolysed capped RNA efficiently, whereas homodimeric AvrM14-A^{E82Q} did not (Fig. 3b). Intriguingly, the product produced by the homodimers migrated to a lower position following separation by thin-layer chromatography (TLC), compared with the product released by the monomers (Fig. 3b). To identify the products released by the mRNA-decapping proteins, we measured the migration of m^7GMP , m^7GDP , GMP and GDP standards using UV shadowing under our TLC conditions (Fig. S7). The Rf values recorded for the m^7GMP , m^7GDP , GMP and GDP standards were used to place the labels on Figs 3(b) and 4(d). Our data indicate that the

major product released by homodimeric AvrM14 is m^7GDP , whereas the major product released by monomeric protein is m^7GMP . Thus, mRNA decapping by homodimeric AvrM14 generates 5'-monophosphate RNA (p-RNA), whereas monomeric AvrM14 generates 5'-diphosphate RNA (pp-RNA) (Fig. 3c). To promote mRNA decay, decapping enzymes are hypothesised to release p-RNA *in vivo*, that can be degraded by 5'-3' exoribonucleases, whereas pp-RNA is resistant to exoribonuclease degradation (Fujimura & Esteban, 2010; Schoenberg & Maquat, 2012; Grudzien-Nogalska & Kiledjian, 2017). Our *in vitro* results therefore suggest that to promote host mRNA decay, AvrM14 would function as a homodimer.

To understand how the AvrM14 proteins homodimerise, we sought to determine the crystal structure of homodimeric AvrM14-B. To ensure any differences observed in the structure were independent of the crystallisation process, we crystallised homodimeric AvrM14-B in identical conditions to monomeric AvrM14-B. The crystal structure demonstrates that homodimerisation is mediated by domain swapping (Fig. 3d). The

Fig. 3 Homodimerisation of AvrM14 is mediated by domain swapping, which alters RNA cap-cleavage location, compared with the monomer. (a) Size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) was used to determine the molecular mass of the different oligomeric forms of AvrM14-B in solution. For all plots, the black lines refer to the right y-axis and indicate the protein elution traces (normalised refractive index) for proteins eluted from the Superdex S75 10/300 increase column. The red bands correspond to the average molecular-mass distribution across the refractive index peak, all molecular masses refer to the left y-axis. The green and purple dashed lines indicate the theoretical molecular mass of monomeric (16.6 kDa) and homodimeric (33.2 kDa) AvrM14 protein, respectively. (b) The separate monomeric and homodimeric forms of AvrM14-A and AvrM14-B were incubated with $m^7Gp^{32}pp$ -RNA and the reaction products were separated using thin-layer chromatography. *HsNudt16* was included as a positive control. The predicted identity of each ^{32}P -labelled compound is indicated on the left, based on the Rf value determined for standard solutions of m^7GMP and m^7GDP . (c) The pyrophosphate bond predominantly hydrolysed by monomeric and homodimeric AvrM14 proteins based on the RNA cap-cleavage assay results. (d) Ribbon model of the crystal structure of homodimeric AvrM14-B (PDB ID: 8DPA), showing C-terminal domain swapping. Chain A is coloured green and chain B purple.

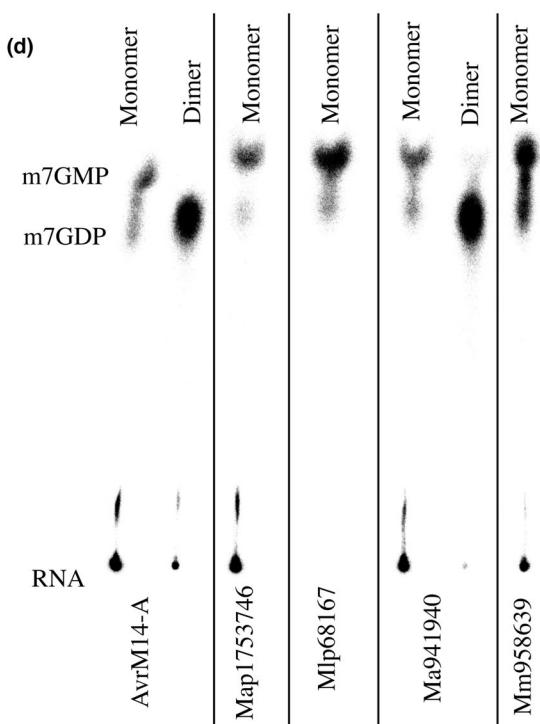
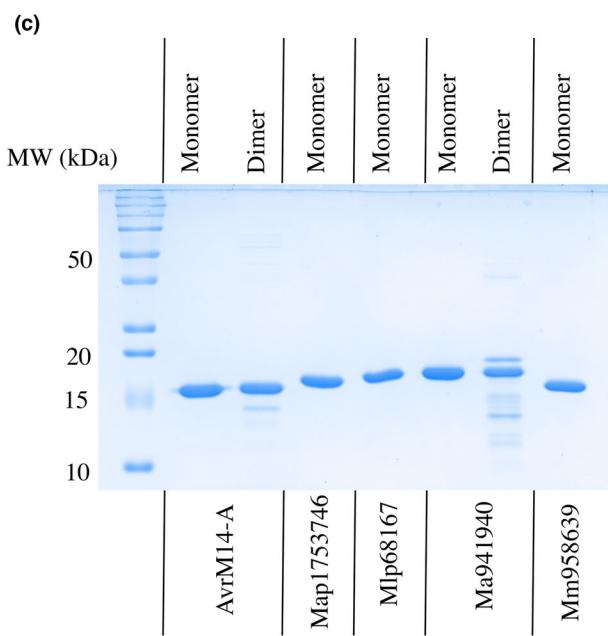
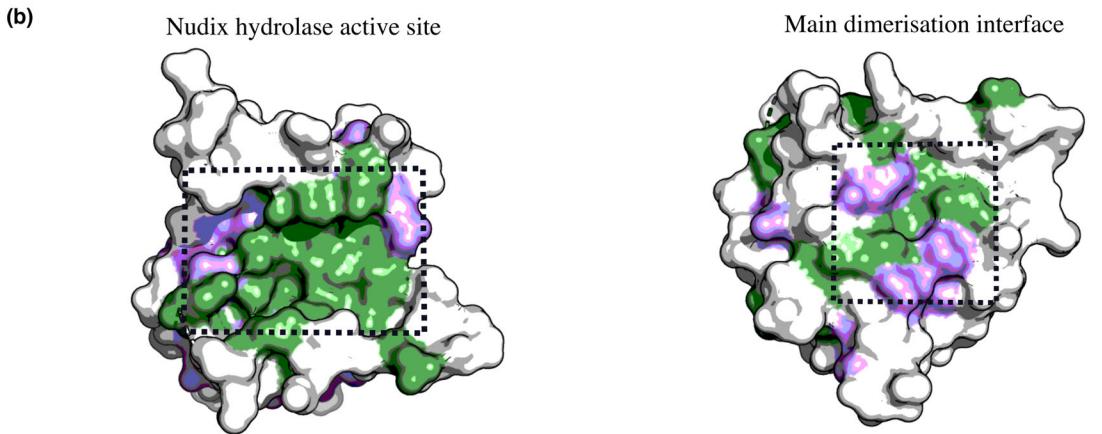
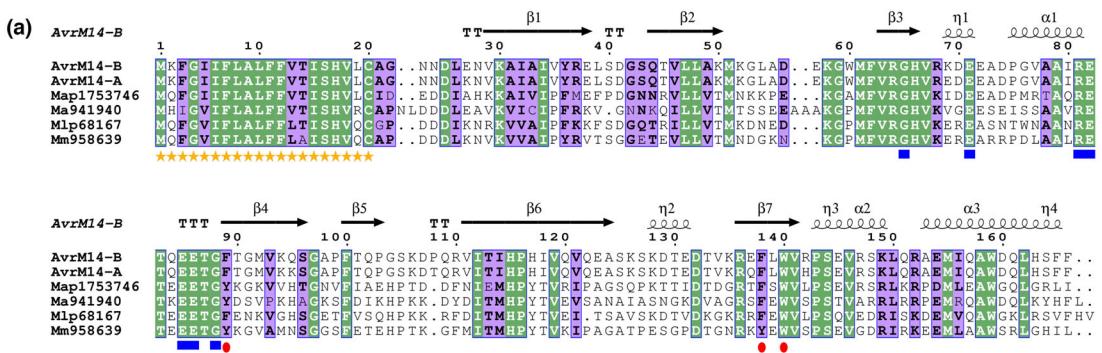


C-terminal regions (residues 124 to 166) from each of the monomeric proteins swap positions to form an intertwined homodimer (Fig. 3d). Domain-swapped homodimeric structures have been reported previously for ADPR-specific Nudix hydrolases (Gabelli *et al.*, 2001; Kang *et al.*, 2003; Yoshioka *et al.*, 2004; Zha *et al.*, 2006; Wakamatsu *et al.*, 2008; Tang *et al.*, 2015). However, the position of each monomeric subunit in the AvrM14 structure differs substantially from the previously reported domain-swapped Nudix hydrolases. The overall positioning of each monomeric subunit and the corresponding dimerisation interface are almost identical to the crystal packing observed in the asymmetric units of monomeric AvrM14-A and AvrM14-B and therefore are similar to the *HsNudt16/X29* homodimers (Figs 3d, S4c, S5). Buried in the main interface between the two monomeric subunits is an aromatic core composed of F⁸⁹, F¹³⁸ and W¹⁴⁰ from both monomers, which we hypothesise promote self-association (Fig. S4c). We attempted to mutate these amino acids to prevent homodimerisation for further experiments; however, the mutant proteins were not stable when expressed in *E. coli*.

To investigate whether stable dimeric forms of AvrM14 are produced *in planta*, we performed native protein extractions from *N. benthamiana* tissue expressing untagged AvrM14 protein. We separated these proteins using SEC, followed by western blot analysis with specific polyclonal antibodies raised against AvrM14 protein (Fig. S8). Using this approach, we observed AvrM14 protein elution from SEC at a volume consistent with monomeric forms of the protein. These data suggest that AvrM14 does not form stable dimers *in planta*, mediated by the domain-swapping mechanism observed in *E. coli*. However, these results do not preclude the potential for AvrM14 to function as a homodimer via transient association with other proteins that cannot be detected using these methods.

RNA-decapping activity is conserved in Nudix effectors from other pathogenic *Melampsora* spp.

We next wanted to determine whether other plant pathogens secrete similar Nudix hydrolase effectors with RNA-decapping activity. BLAST searches with JGI and NCBI databases yielded



proteins homologous to AvrM14 from all genome-sequenced *Melampsora* species. Their sequence identity to the AvrM14 proteins is c. 50%, with all sequences demonstrating conservation of the signal peptide, Nudix box and residues involved in the

dimerisation interface (Fig. 4a). Mapping the sequence conservation onto the structure of monomeric AvrM14-B demonstrates extremely high conservation around the Nudix hydrolase active site, suggesting that the homologues are active Nudix hydrolases

Fig. 4 Predicted effectors homologous to AvrM14 from multiple *Melampsora* species decap RNA *in vitro*. (a) Sequence alignment (produced using ESPRIPT 3.0) of AvrM14 variants and homologous proteins from other *Melampsora* species (Robert & Gouet, 2014). Each sequence is labelled with the relevant unique protein ID from the JGI database and the first letter of their genus and species name (Mm for *Melampsora medusae*, Mlp for *Melampsora larcini-populina*, Map for *Melampsora allii-populina*, and Ma for *Melampsora americana*). Residues with 100% conservation are coloured green, residues with conserved substitutions are coloured purple, white are nonconserved residues, the predicted signal peptide region is indicated by orange stars, the aromatic residues in the AvrM14 dimeric interface are indicated by red dots, and the conserved Nudix box residues are indicated by blue squares. Above the sequence alignment is the secondary structure of AvrM14-B (black arrows indicating β -strands, squiggles indicating helices, and the TT and TTT symbols indicating β -turns and α -turns respectively). (b) The structure of monomeric AvrM14-B, demonstrating the surface regions with the highest conservation across the homologues. Residue colouring is the same as in the sequence alignment. The Nudix hydrolase active site and the major interface involved in dimerisation are indicated. (c) Coomassie-stained SDS-PAGE analysis demonstrating the purity of each protein used in the RNA-decapping assays. (d) Recombinant AvrM14-A and the homologous proteins were incubated with m7 Gp 32 pp-RNA and the reaction products were separated using thin-layer chromatography. The oligomeric state of each protein, as predicted by size-exclusion chromatography, is indicated along the top. The predicted identity of each 32 P labelled compound is indicated on the left, based on the Rf value determined for standard solutions of m7 GMP and m7 GDP. A middle lane not relevant to the analysis has been removed from the image.

featuring similar substrate specificity (Fig. 4b). The surface involved in dimerisation is also highly conserved (Fig. 4b). No Nudix hydrolases with similarity to AvrM14 were identified outside of the genus *Melampsora*.

The predicted AvrM14 homologues from *Melampsora americana*, *Melampsora medusae*, *Melampsora allii-populina* and *Melampsora larcini-populina* were selected for biochemical analysis (sequences in Table S1). Size-exclusion chromatography indicated that all homologues were predominantly in a monomeric state, following purification from *E. coli* (Fig. S9). All monomeric proteins could be purified to near homogeneity and decapped mRNA *in vitro* (Fig. 5). The assay results demonstrate that RNA-decapping activity has been conserved throughout the evolution of this gene family in *Melampsora* spp. The AvrM14 homologues from all species, except *M. medusae*, also showed a SEC absorbance peak at a position consistent with a homodimeric protein (Fig. S9). However, low yields of the putative homodimeric proteins meant that only one (the homologue from *M. americana*) could be purified (Fig. 4c). Analogous to homodimeric AvrM14 from *M. lini*, the homologous *M. americana* homodimer released predominantly m7 GDP from capped RNA *in vitro*, whereas the monomeric protein released predominantly m7 GMP (Fig. 4d).

AvrM14 expression in flax promotes the expression of genes involved in growth and metabolic processes while inhibiting aspects of plant immunity

To better characterise how the AvrM14 effector impacts host plant physiology, we transiently expressed AvrM14-A in flax leaves and conducted transcriptome-wide RNA-sequencing (RNA-Seq) analysis. The RNA was extracted from leaves recently infiltrated with *Agrobacterium*, to enable AvrM14 expression. Therefore, our data are representative of the changes induced by AvrM14 following the activation of plant immune responses by *Agrobacterium* exposure. Our differential expression (DE) analysis identified 488 and 312 significantly (q value <0.05) more abundant ($\log_2\text{fc} > 0.5$) and less abundant ($\log_2\text{fc} < -0.5$) transcripts, respectively, in leaves agroinfiltrated with *AvrM14-A*, compared with the vector-only control (Table S4). Gene Ontology (GO) enrichment analysis indicates that AvrM14 expression promotes the expression of genes involved in carbon fixation, photosynthetic reactions and various metabolic processes (Fig. 5a), and suppresses the expression of genes involved in cell-

cell recognition, hypoxia, salt and biotic stress responses (Fig. 5a). To identify which changes are due to the enzymatic activity of AvrM14, we also conducted RNA-Seq on flax leaves expressing *AvrM14-A^{E82Q}* and compared the *AvrM14-A* and *AvrM14-A^{E82Q}* datasets. Both *AvrM14-A* and *AvrM14-A^{E82Q}* were expressed at similar levels (Fig. S10). The DE analysis indicates that many of the differences between the *AvrM14-A* and vector-only datasets are unrelated to enzymatic activity, with only 90 significantly more abundant and 36 significantly less abundant transcripts in leaves agroinfiltrated with *AvrM14-A*, compared with *AvrM14-A^{E82Q}* (Table S5). There is multiple significantly less abundant transcripts homologous to pseudo-response regulator 5 (APRR5) from *A. thaliana* (Fig. 5b). Consistent with changes to APRR5 levels, there is an overrepresentation of transcripts homologous to *A. thaliana* APRR5 targets (Nakamichi *et al.*, 2012) in the significantly less abundant transcripts (Fig. 5c). APRR5 is an important regulator of *A. thaliana* circadian rhythm (Nakamichi *et al.*, 2005; Rawat *et al.*, 2011); our results therefore suggest that the enzymatic activity of AvrM14 effectors could impact circadian rhythm processes.

Surface-exposed residues required for M1 and M4 recognition

In addition to characterising the virulence function of AvrM14, we were interested in understanding the requirements for recognition of AvrM14 by M1 and M4. Flax M1 belongs to the TIR-NLR class of R-proteins. M4 has not yet been cloned, but is expected to encode a protein closely related to M1 (Lawrence *et al.*, 2010).

The AvrM14 structures reveal that the six amino acid polymorphisms differentiating the A and B alleles map to the protein surface (Fig. 6a). To understand which polymorphisms are important for recognition, we transiently expressed various AvrM14 proteins with single or double mutations at the six polymorphic residues in tobacco co-expressing *M1* (*M1* tobacco) and in near-isogenic lines of the flax variety Bison containing either *M1* (*M1* flax) or *M4* (*M4* flax), using *Agrobacterium*-mediated transformation (sequences in Table S1).

The expression and accumulation of all mutant AvrM14 proteins in plant tissue were confirmed via Western blotting (Fig. S2). Most of the mutations had only a minor or no effect on the observed phenotype (Figs S11, S12; Table S6). Only by

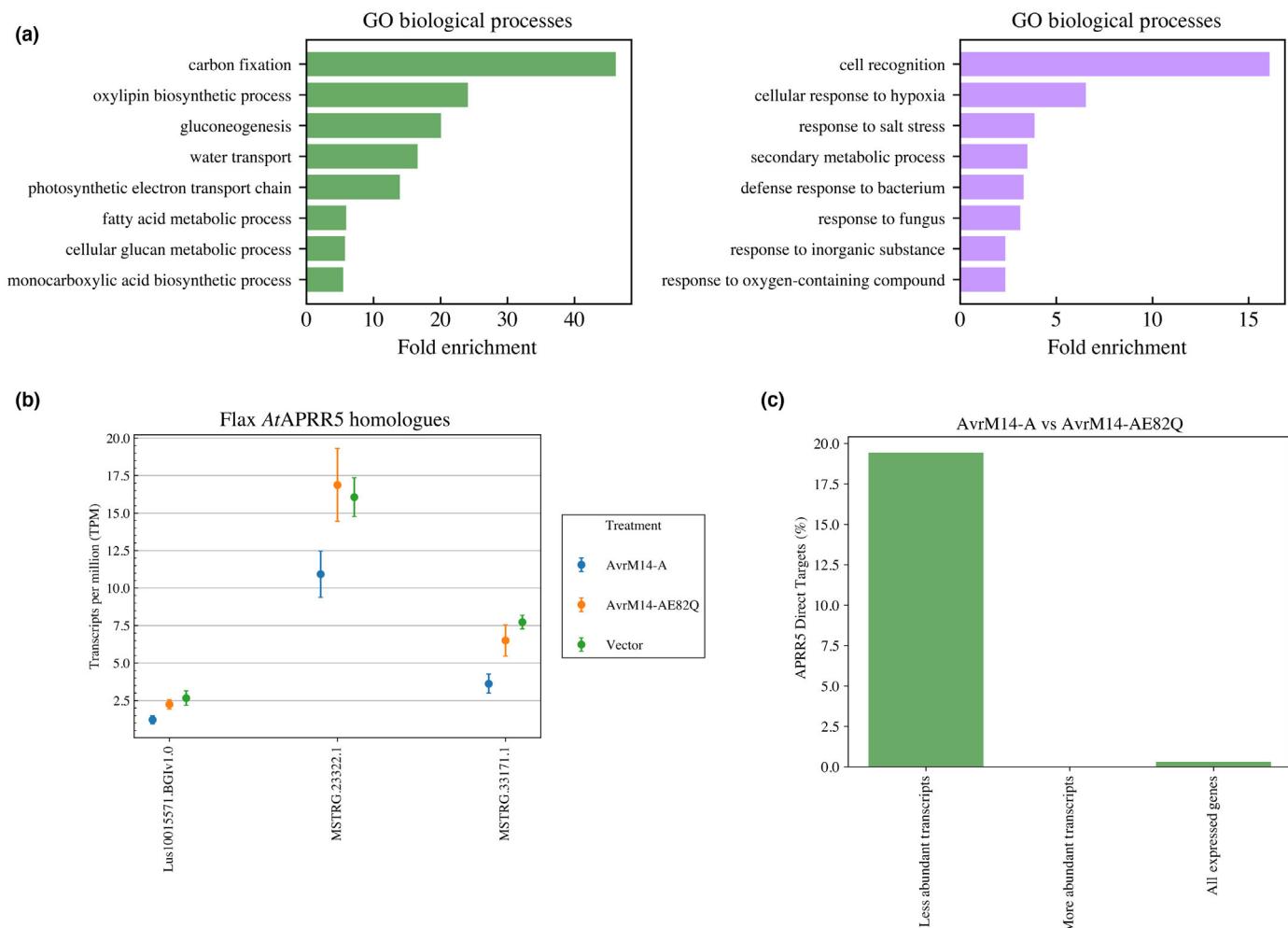


Fig. 5 Enzymatic activity of AvrM14-A in flax leaves reduces the relative abundance of transcripts homologous to *Arabidopsis thaliana* APPR5 and APPR5 direct targets. (a) The Gene Ontology biological processes that are enriched ($FDR < 0.05$) in the list of transcripts significantly ($q < 0.05$) more abundant (left, green; $\log_2 fc > 0.5$) and less abundant (right, purple; $\log_2 fc < -0.5$), when comparing flax leaves transformed with AvrM14-A to an empty vector. Only the top 10 (sorted by fold enrichment) processes are shown, after filtering out redundant terms using REVIGO (Supek *et al.*, 2011). (b) The average expression levels ($\pm SDs$) are displayed as transcripts per million (TPM) for transcripts homologous to *A. thaliana* PRR5 that are significantly less abundant ($q < 0.05$, $\log_2 fc < -0.5$) when comparing the AvrM14-A (blue) ($n = 4$) and AvrM14-AE82Q (orange) ($n = 4$) RNA-Seq datasets, the mean TPM ($\pm SDs$) are also displayed for the vector-only dataset (green) ($n = 3$). The x-axis label indicates the name of the transcript as listed in Supporting Information Tables S4 and S5. (c) The percentage of transcripts homologous to *A. thaliana* APPR5 direct targets, as identified by Nakamichi *et al.* (2012), present in the list of transcripts significantly ($q < 0.05$) less abundant (left; $\log_2 fc < -0.5$), or more abundant (middle) ($\log_2 fc > 0.5$) when comparing flax leaves expressing AvrM14-A to leaves expressing AvrM14-AE82Q. The percentage of transcripts homologous to *A. thaliana* APPR5 direct targets present among all expressed genes included in the differential expression analysis is indicated on the right.

mutating residues 95 and 108, we were able to completely and consistently reverse recognition by both *M1* and *M4* in flax (i.e. AvrM14-A^{K95Q/T108P} expression resulted in a HR similar to AvrM14-B expression and AvrM14-B^{Q95K/P108T} expression resulted in a HR similar to AvrM14-A expression; Fig. 6c; Table S6).

Overall, our results indicate that both *M1* and *M4* R-proteins recognise AvrM14-A using a mechanism that can be evaded by mutating residues 95 and 108, suggesting that the currently uncharacterised *M4* is highly similar to *M1*, consistent with the observation that *M1* and *M4* are likely to be allelic (Lawrence *et al.*, 2010). According to the crystal structure of monomeric and homodimeric AvrM14, residues 95 and 108 do not localise to the same surface region (Fig. 6b). If *M1* and *M4* interact

directly with AvrM14, our results suggest that the R-proteins have multiple contact points with the effector, like previous data suggest occurs with other flax/flax rust R-protein/effector pairs, AvrM and M, and AvrL567 and L5/L6 (Ravensdale *et al.*, 2012; Ve *et al.*, 2013).

Discussion

In this study, we have demonstrated that the AvrM14 effector from *M. lini* is a Nudix hydrolase that is able to suppress plant immunity, modify host transcriptome composition and uncap mRNAs in a manner that would render them susceptible to degradation by host exoribonucleases. No plant pathogens have been previously reported to use mRNA-decapping enzymes to

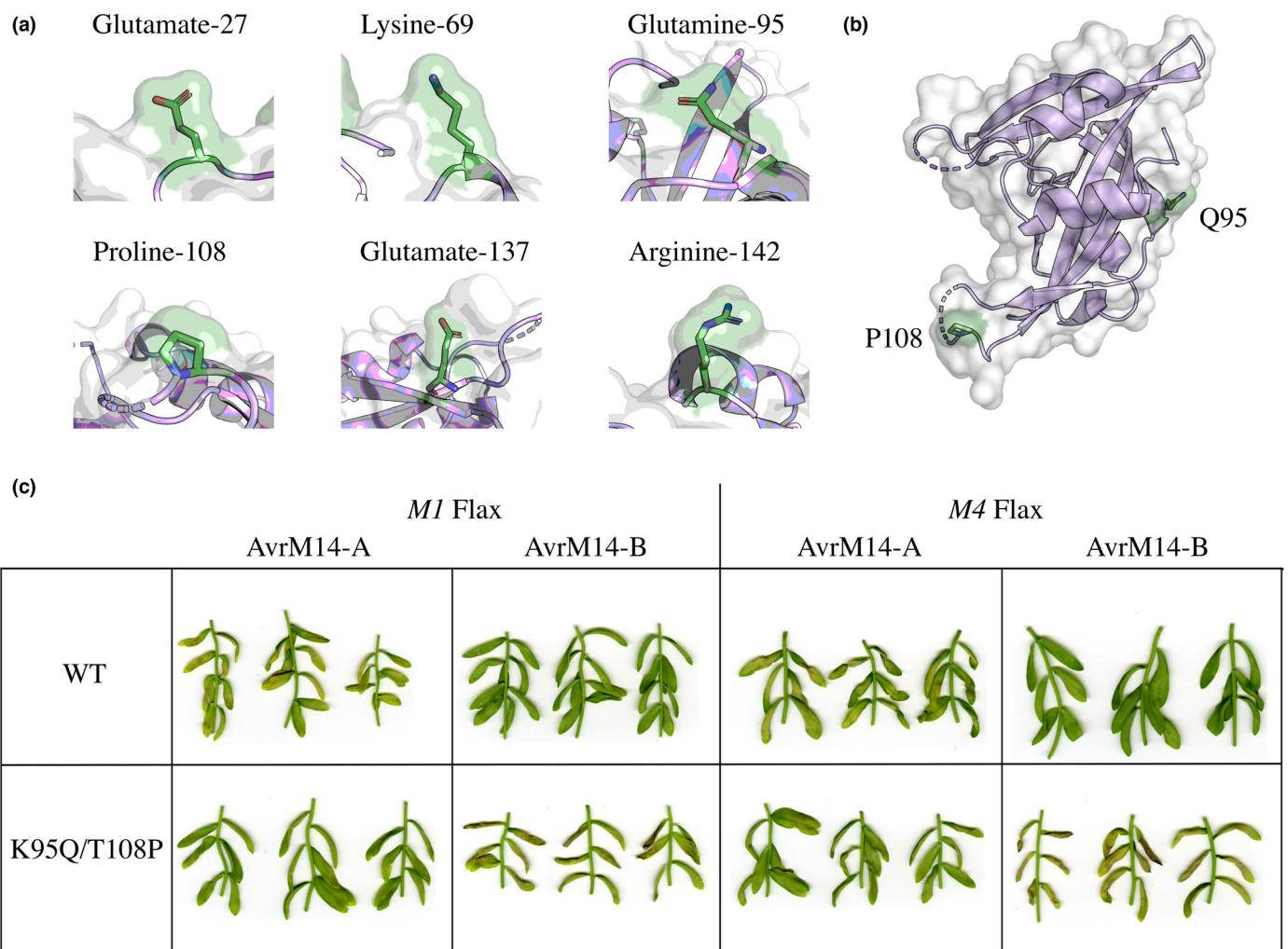


Fig. 6 AvrM14 polymorphic residues are surface-exposed, with residues 95 and 108 involved in resistance protein recognition. (a) The six polymorphic residues differentiating AvrM14-A and AvrM14-B all localise to the protein surface, as demonstrated here using the AvrM14-B structure displayed in ribbon representation, superimposed on a surface representation, with side chains of polymorphic residues coloured green. (b) The crystal structure of AvrM14-B with the side chains of residues important for evading M1 and M4 detection (Q95 and P108) coloured green. (c) Bison \times M1 (Williston Brown) or M4 (Victory 'A') flax (*Linum usitatissimum*) lines were infiltrated with *Agrobacterium* harbouring constructs encoding either wild-type or mutant YFP-tagged AvrM14 proteins (AvrM14-A, AvrM14-B, AvrM14-A^{K95Q/T108P} or AvrM14-B^{Q95K/P108T}). The agroinfiltrated leaves were photographed 5 d post-infiltration to assess the cell-death response.

inhibit plant immunity. Before the present study, only certain animal viruses, including vaccinia and African swine fever viruses, have been known to produce mRNA-decapping Nudix hydrolases during host-cell infection (Parrish & Moss, 2006; Quintas *et al.*, 2017). Our findings have implications for understanding genetic post-transcriptional regulation in plants, in addition to helping understand how *Melampsora* pathogens circumvent plant immunity during successful infection.

Host and pathogen Nudix hydrolases influence plant immunity

Diverse pathogens of plants secrete effectors with Nudix domains and prior research have confirmed that two of these (Avr3b and RipN) can inhibit plant immune function (Dong *et al.*, 2011; Dong & Wang, 2016; Sun *et al.*, 2019). We have demonstrated

that a third effector, AvrM14 from flax rust, is also a Nudix hydrolase that can suppress plant immunity. The Nudix hydrolase activity of AvrM14 inhibits the MAMP-induced ROS burst in the nonhost plant *N. benthamiana* and reduces the HR cell-death response triggered by M1 in flax. However, it is currently unknown whether the suppressive effect of AvrM14 on plant immunity promotes the virulence of flax rust during infection.

Plant Nudix hydrolases also play important roles in regulating plant immune responses. *Arabidopsis thaliana* Nudix hydrolases 6, 7 and 8 (AtNUDX6/7/8) have all been implicated in modulating plant immune function (Bartsch *et al.*, 2006; Ge *et al.*, 2007; Ishikawa *et al.*, 2010; Fonseca & Dong, 2014). Additionally, wheat Nudix hydrolase 23 (TaNUDX23) suppresses ROS accumulation and is stabilised by an effector protein from *Puccinia striiformis* f. sp. *tritici* (Yang *et al.*, 2020). Recently, it was reported that AtNUDX7 and the Avr3b effector can degrade

cyclic nucleotide monophosphates (cNMPs) by acting as phosphodiesterases (Yu *et al.*, 2022). Yu *et al.* (2022) demonstrated that cNMPs are important for TIR-NLR-mediated plant immunity and that NUDX7 can suppress the cell-death response triggered by the TIR-only disease R-protein RBA1 (Yu *et al.*, 2022). AvrM14 does not hydrolyse similar substrates to AtNUDX7, TaNUDX23 or Avr3b *in vitro* and is not structurally similar to AtNUDX7 (Tang *et al.*, 2015); our data support a different mechanism of immunity suppression.

AvrM14 is a highly selective Nudix hydrolase

Nudix hydrolases are renowned for their broad substrate specificity, with many enzymes readily hydrolysing multiple substrates *in vitro* (McLennan, 2013). Avr3b, AtNUDX7, TaNUDX23 and RipN were tested against a limited subset of potential substrates and all hydrolysed multiple substrates (Ogawa *et al.*, 2005; Dong *et al.*, 2011; Sun *et al.*, 2019; Yang *et al.*, 2020), complicating the identification of physiologically relevant substrates. By contrast, we found that AvrM14 does not exhibit broad substrate specificity *in vitro*, based on extensive screening of a total of 70 potential substrates. AvrM14 only hydrolysed mRNA cap analogues and capped mRNA. In our broad screen, AvrM14 effectively hydrolysed m^7 Gp₅G but not Gp₅G, suggesting that AvrM14 achieves substrate specificity by selecting for the methylated guanosine. Nudix hydrolases with specificity for methylated guanosine have been reported previously and experimental evidence indicates that these proteins function as mRNA-decapping enzymes (Van Dijk *et al.*, 2002; Wang *et al.*, 2002; Piccirillo *et al.*, 2003; Parrish *et al.*, 2007; Parrish & Moss, 2007). The canonical mRNA-decapping enzyme in eukaryotes, DCP2, demonstrates improved mRNA-decapping activity with m^7 Gp₃RNA, when compared to Gp₃RNA (Van Dijk *et al.*, 2002; Wang *et al.*, 2002; Piccirillo *et al.*, 2003). Additionally, two mRNA-decapping enzymes from the vaccinia virus, D9 and D10, both demonstrate specificity for methylated guanosine substrates (Parrish *et al.*, 2007; Parrish & Moss, 2007). Structural studies with mRNA cap analogues indicate that the N-terminal regulatory domain (NRD) of DCP2 is important for binding to the cap region (Charenton *et al.*, 2016; Mugridge *et al.*, 2016; Wurm *et al.*, 2017). AvrM14 lacks an NRD, therefore binding cannot be controlled by the same mechanism as DCP2. We attempted cocrystallisation of AvrM14 with the mRNA cap analogues m^7 Gp₃G and m^7 Gp₅G to understand the molecular basis of substrate selectivity, but were unable to capture them bound to the enzyme.

AvrM14 likely functions as a homodimer to ensure the correct mRNA cap pyrophosphate bond is hydrolysed

We found that a proportion of AvrM14 protein was produced in *E. coli* as a stable dimer and demonstrated via X-ray crystallography that this was mediated via domain swapping. Dimerisation via domain swapping has been shown previously for other Nudix hydrolases (Gabelli *et al.*, 2001; Kang *et al.*, 2003; Yoshioka *et al.*, 2004; Zha *et al.*, 2006; Wakamatsu *et al.*, 2008; Tang

et al., 2015), and we were interested in investigating the biological relevance of this, if any, for AvrM14. Our *in planta* experiments show that AvrM14 is monomeric when expressed in *N. benthamiana*, indicating that homodimerisation of AvrM14 via the domain-swapping mechanism in plants is unlikely. Unfortunately, the method we used to discriminate monomeric and homodimeric AvrM14 is not sensitive enough to investigate what the fungus itself delivers into flax cells during infection. Despite this, we suggest that AvrM14 functions as a dimer to decap mRNA. Our *in vitro* and *in silico* analyses provide multiple lines of evidence for AvrM14 functioning as a homodimer. We demonstrate that homodimeric AvrM14 releases m^7 GDP and 5'-monophosphate RNA from capped mRNA. It is well established that following decapping, mRNA transcripts in cells are subject to 5'-3' decay by exoribonucleases (XRN4 in plants), which target a 5' end monophosphate RNA substrate, and that 5' diphosphate RNA is not a direct target for 5' decay (Grudzien-Nogalska & Kiledjian, 2017). In support of this, the structurally related decapping enzyme Nudt16, homodimerises and preferentially releases m^7 GDP (Song *et al.*, 2010, 2013; Trésagues *et al.*, 2015). There are also strong similarities between the homodimerisation interface observed in the Nudt16 crystal structure (PDB ID: 3MGM) and the crystal packing, and domain-swapping dimer, observed for AvrM14-A and AvrM14-B. In addition, the aromatic residues involved in the dimeric interface of AvrM14 are highly conserved in homologous effectors from other *Melampsora* species, suggesting that this protein–protein interaction surface is important for effector function. Collectively, based on our *in vitro* and *in silico* analysis of AvrM14 and the homologous effectors, we suggest that these proteins are biologically active as dimers, but how this is achieved in a natural setting, that is domain swapping, interactions with other proteins or mRNA itself, remain unknown.

Controlling plant immunity by decapping mRNA

AvrM14 decaps mRNA *in vitro* and the mRNA-decapping activity requires glutamate-82 (E82). We also demonstrated that E82 is required for suppression of ROS production in *N. benthamiana* and for the reduction in the cell-death response following AvrM14 recognition by M1 in flax. Our results are consistent with AvrM14 acting as an mRNA-decapping enzyme, to inhibit aspects of plant immunity. This suggests that AvrM14 can selectively decap transcripts involved in plant immunity, rather than destabilising the entire transcriptome. We speculate that AvrM14 may interact with other proteins in the plant cell to enable transcript selectivity, as occurs with DCP2 in yeast (He *et al.*, 2018). Alternatively, AvrM14 may be hydrolysing another unknown compound *in planta* that also requires E82 to inhibit plant immunity. However, due to the selectivity of AvrM14 for methylated guanosine substrates *in vitro*, our substrate screening data suggest that this is unlikely.

The decapping and decay of plant mRNA are known to be important for plant immune function. For example, following immune activation, phosphorylation of the DCP2-interacting protein DCP1 (decapping 1) enhances DCP2-dependent decay

of multiple transcripts encoding negative regulators of plant immunity (Yu *et al.*, 2019; He *et al.*, 2022). By contrast, during periods of normal growth, mRNA decapping and decay can prevent immune activation via nonsense-mediated mRNA decay (NMD) of numerous *R*-gene transcripts (Gloggnitzer *et al.*, 2014; Jung *et al.*, 2020). Nonsense-mediated mRNA decay regulation for many of these *R*-genes requires VARICOSE (VCS), a scaffolding protein for decapping machinery (Xu *et al.*, 2006; Raxwal *et al.*, 2020).

Our RNA-Seq analysis identified that the enzymatic activity of AvrM14-A decreases the abundance of multiple transcripts homologous to *A. thaliana* circadian network-related genes. Plant defence responses, including ROS production and the HR triggered by R-proteins, are linked to circadian rhythm (Lu *et al.*, 2017; Westwood *et al.*, 2019; Butt *et al.*, 2020). For example, the expression of some *A. thaliana* *R*-genes is controlled by the circadian regulator, CIRCADIAN CLOCK-ASSOCIATED 1 (Wang *et al.*, 2011) and the amplitude of ROS production following flg-22 exposure is time-of-day-dependant (Korneli *et al.*, 2014). The suppression of ROS production and reduction in the M1-activated HR by the enzymatic activity of AvrM14 may be a result of AvrM14 altering plant circadian rhythm.

The transcript level of regulators of circadian rhythm in *A. thaliana* has recently been identified to be controlled by 5'-3' mRNA decay requiring the cytoplasmic exoribonuclease XRN4 (Careno *et al.*, 2022). We speculate that AvrM14 decreases the abundance of flax circadian rhythm regulator transcripts by removing their 5' cap and promoting their XRN4-dependant decay. Further research is required to confirm that the decrease in the abundance of APRR5 and other circadian-rhythm-related transcripts due to the enzymatic activity of AvrM14 is a direct result of these transcripts being decapped by AvrM14.

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Competing interests

None declared.

Author contributions

CLM, A-MC, PND, BK, DAJ and SJW planned and designed the research study. CLM, A-MC, JRG, AMD, MAO, DSY and SJW performed the experiments; CLM, A-MC, JRG, DJE, SEB, PND, DAJ and SJW analysed the data. CLM wrote the original draft and all authors contributed to writing, reviewing and editing of the manuscript. CLM and A-MC contributed equally to this work.

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Data availability

The data that support the protein structures described in this study are openly available under accession nos. 8DP8, 8DP9 and 8DPA at the PDB. RNA-sequencing data are available from the NCBI (GEO: GSE207874, SRA: SRX16112399, SRX16112400, SRX16112401, SRX16112402, SRX16112403, SRX16112404, SRX16112405, SRX16112406, SRX16112384, SRX16112385 and SRX1611238).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Structural features of AvrM14.

Fig. S2 Western blots demonstrating the expression and accumulation of the AvrM14 wild-type (WT) and mutant proteins in agroinfiltrated plant tissue.

Fig. S3 Mutation of Nudix-box glutamate E82 to glutamine does not alter the recognition of AvrM14-A or AvrM14-B by M1 when co-expressed in tobacco.

Fig. S4 Recombinant AvrM14 proteins elute as two distinct peaks during size-exclusion chromatography (SEC); all AvrM14 crystal structures display similar self-association interfaces.

Fig. S5 AvrM14-B crystal packing dimer (A, purple) compared with homodimeric *Homo sapiens* Nudt16 (PDB ID: 3MGM) (B, gold) with the Nudix helices coloured green.

Fig. S6 Screening of common Nudix hydrolase substrates using monomeric and homodimeric AvrM14 protein.

Fig. S7 Migration and Rf values for standard solutions of potential mRNA-decapping products.

Fig. S8 Identifying stable multimeric AvrM14 protein complexes from *Nicotiana benthamiana* protein extracts.

Fig. S9 Size-exclusion chromatograms displaying the elution profile of each of the AvrM14 homologues assessed in our study.

Fig. S10 Average expression level (\pm SD) in transcripts per million for AvrM14 genes in each of the RNA-sequencing datasets.

Fig. S11 Co-expression of AvrM14 wild-type and mutant proteins with M1 in tobacco.

Fig. S12 Expression of wild-type and mutant AvrM14 proteins in M1 and M4 flax.

Methods S1 Extended Materials and Methods.

Table S1 DNA and protein sequences used in this study.

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Table S3 Substrates and secondary enzymes used in the phosphate sensing fluorophore-based hydrolysis assays with recombinant AvrM14-A.

Table S4 Average read counts with standard deviations in TPM as determined by Salmon, the differential expression (DE) analysis output (\log_2 fc and *q*-values) as determined by fishpond when comparing the AvrM14-A and vector-only datasets and the BLAST-2-Go best hit for all transcripts.

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New Phytologist Supporting Information

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Authors: Carl L. McCombe, Ann-Maree Catanzariti, Julian R. Greenwood, Anna M. Desai, Megan A. Outram, Daniel S. Yu, Daniel J. Ericsson, Steven E. Brenner, Peter N. Dodds, Bostjan Kobe, David A. Jones and Simon J. Williams

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The following Supporting Information is available for this article:

Extended Materials and Methods

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Extended Materials and Methods:

Protein production

Recombinant *HsNudt16* protein was purchased from Abcam (Cambridge, United Kingdom). All other proteins were expressed in *E. coli* BL21 (DE3) cells using ZYP-5052 (AvrM14 wild-type and mutant proteins) or ZYM-5052 (*Melampsora* homologues) autoinduction media (Studier, 2005). Cells were grown by continuous shaking at 37°C until the OD_{600nm} reached 0.6 – 0.8. The temperature was then dropped to 18°C and incubated with shaking for another 18 hours. The cells were harvested by centrifugation and resuspended in lysis buffer containing 50 mM HEPES pH 7 (Ma941940, Mlp68167) or 8 (AvrM14-A, AvrM14-B, AvrM14-A^{E82Q}, AvrM14-B^{E82Q}, Map1753746, Mm958639), 150 mM NaCl, and 1 mM DTT. Cells were lysed using sonication and cellular debris pelleted by centrifugation. The resulting supernatant was applied to a 5 mL HisTrap FF crude column (Cytiva, Marlborough, Massachusetts). To remove loosely bound proteins, the column was washed with the lysis buffer containing 30 mM imidazole. The remaining bound proteins were eluted with a continuous gradient of imidazole from 30 mM to 250 mM over 10 minutes, using an Äkta pure chromatography system. Fractions were analysed by Coomassie-stained SDS-PAGE and fractions containing the protein of interest were pooled and buffer exchanged to remove the imidazole before the addition of recombinant 6xHis-tagged TEV (AvrM14 proteins) or 3C protease (*Melampsora* homologs) for overnight tag cleavage at 4°C. Protease cleavage was confirmed via SDS-PAGE and the protein solution reapplied to the HisTrap FF column to remove the protease, cleaved 6xHis tag, and any other contaminants. Proteins were then purified further by SEC using a HiLoad 26/600 Superdex® 75 pg column (Cytiva) pre-equilibrated with 10 mM HEPES pH 7 (Ma941940, Mlp68167) or 8 (AvrM14-A, AvrM14-B, AvrM14-A^{E82Q}, AvrM14-B^{E82Q}, Map1753746, Mm958639), 150 mM NaCl, and 1 mM DTT using an Äkta pure chromatography system (Cytiva). After SEC, fractions containing the protein of interest were identified using SDS-PAGE and concentrated using Amicon® Ultra Centrifugal filters (Merck, Darmstadt, Germany) before storage at -80 °C.

In planta oligomeric state analysis (relates to Fig. S8)

N. benthamiana tissue 4-days post agroinfiltration with either the pEG102 AvrM14-A, pEG102 AvrM14-B or a pEG102 empty vector construct, was ground in liquid nitrogen to a fine powder.

The powder was combined with an equal volume of protein extraction buffer (25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2% w/v PVPP, 5 mM DTT, 1 mM PMSF, and 1X cOmplete protease inhibitor) and thoroughly mixed. Insoluble material was pelleted by centrifugation. The supernatant was concentrated to 25% of the starting volume using 10kDa Amicon® Ultra centrifugal filters (Merck). The solution was filtered and then passed over a S75 10/300 analytical size-exclusion chromatography column equilibrated in buffered solution (10 mM HEPES pH 8.0, 150 mM NaCl, and 1 mM DTT). Immunoblot analysis as described above was completed on the elution fractions to detect AvrM14 protein.

RNA sequencing analysis

Reads were aligned to the flax reference genome (*Lusitatissimum_200_BGIv1.0.fa*) (Wang *et al.*, 2012) using Hisat2 (Kim *et al.*, 2019). Sequence alignment map files (.sam) were sorted and converted to binary format (.bam) using samtools (Danecek *et al.*, 2021). Transcripts were assembled using Scallop2 (Zhang *et al.*, 2021). Reference annotations in General Feature Format (.gff3) were converted to General Transfer Format (.gtf) using gffread (Pertea & Pertea, 2020) and were merged with the Scallop2 annotations for all samples using stringtie (Pertea *et al.*, 2015). Unique transcripts that were not present in the reference annotation were identified using gff compare and were separated into a new .gtf file using gtcuff. The sequences of unique transcripts were extracted from the reference genome using gffread and then merged with the reference transcript file. This transcriptome was used for read quantification.

To aid in data analysis, each transcript was compared against the *Arabidopsis* TAIR10 protein list using Blast2GO within OmicsBox (v2.0) to provide a functional annotation (e-value cutoff: 1.0E-25, minimum length of match 50 amino acids) (the best hit for each transcript is available in Supplementary Table S4 and S5).

Salmon (v1.6.0) was used for transcript quantification following recommended procedures for the mapping-based mode (Patro *et al.*, 2017). First, a decoy transcriptome was created using our transcriptome and the entire flax genome (Wang *et al.*, 2012) (v1.0). A Salmon index was then generated using the recommended kmer length of 31. The quant command was used to quantify reads for each dataset using the generated index with validateMappings and gcBias flags activated and numGibbsSamples set to 20. Differential expression analysis was then completed using Swish within the fishpond R package (v1.8.0) (Zhu *et al.*, 2019), utilising tximeta (v1.10) (Love *et al.*, 2020) for data importation, the seed value was set to 1, and only transcripts with at least 10 reads

for three of the replicates for both conditions were analysed, as recommended by the developers (see output log2fc, p- and q-values in Supplementary Tables S4 and S5, transcripts excluded from DE analysis have no values listed in these columns). Gene ontology (GO) enrichment analysis using the Gene Ontology online resource (Ashburner *et al.*, 2000; Consortium, 2020) and Panther Overrepresentation Test (v16) (Mi *et al.*, 2020), was completed on the significantly upregulated (q value < 0.05; log2fc > 0.5) and downregulated (q value < 0.05 and log2fc < -0.5) groups of transcripts (using the gene name of the *Arabidopsis* homologue from the Blast2GO results and *Arabidopsis* as the reference organism). GO biological processes were overrepresented when the FDR was < 0.05. REVIGO (Supek *et al.*, 2011) was used to remove redundant GO terms.

Fig. S1 Structural features of AvrM14. Cartoon representation of the crystal structure of AvrM14-B, with key residues displayed in stick representation. (a) The unusual 3_{10} -helix (red) present in AvrM14's Nudix-box region. (b) The salt bridge observed between E⁷¹ and R⁸¹, with the hydrogen bonds displayed as yellow dashed lines. (c) The putative metal-ion binding glutamates (E⁸², E⁸⁵, E⁸⁶) of AvrM14-B (purple) superimposed onto the structure of a Mg²⁺ bound ADPR-hydrolysing Nudix enzyme (green; PDB ID: 1KHZ).

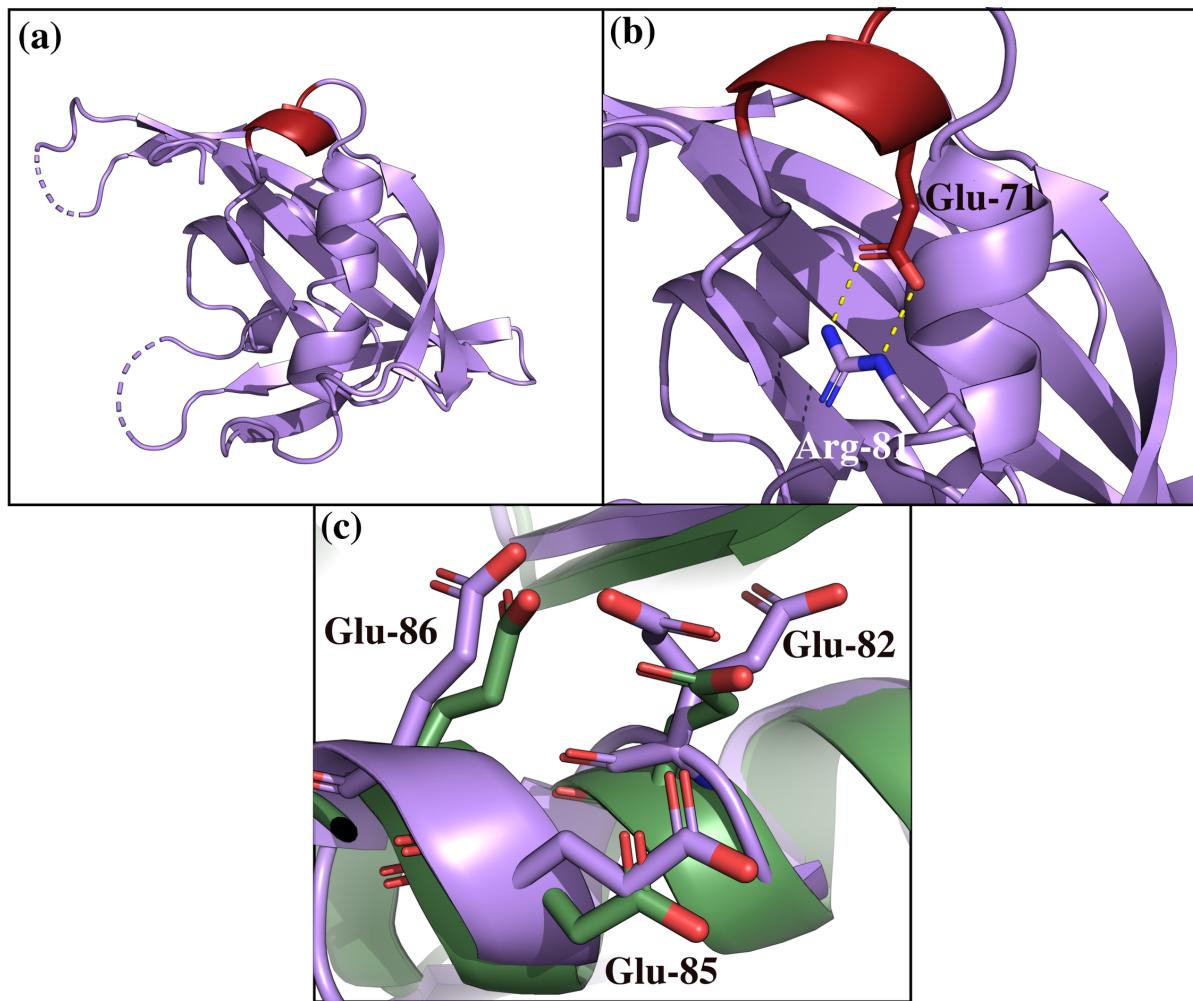


Fig. S2 Western blots demonstrating the expression and accumulation of the AvrM14 wild-type (WT) and mutant proteins in agroinfiltrated plant tissue. Total protein extracts from *Nicotiana tabacum* leaf tissue agroinfiltrated with a construct to express a YFP-tagged AvrM14 protein or with an empty pEG104 vector, were analysed by western blotting. Mouse anti-GFP (Roche) and goat anti-mouse-HRP (Amersham) were used as the primary and secondary antibodies, respectively. Ponceau S staining shows protein loading. The numbers on the left of each blot indicate the sizes of molecular weight markers. The top row of blots are AvrM14-A protein variants, and the bottom row of blots are AvrM14-B protein variants, the expected size of YFP-tagged AvrM14 is approximately 44 kDa.

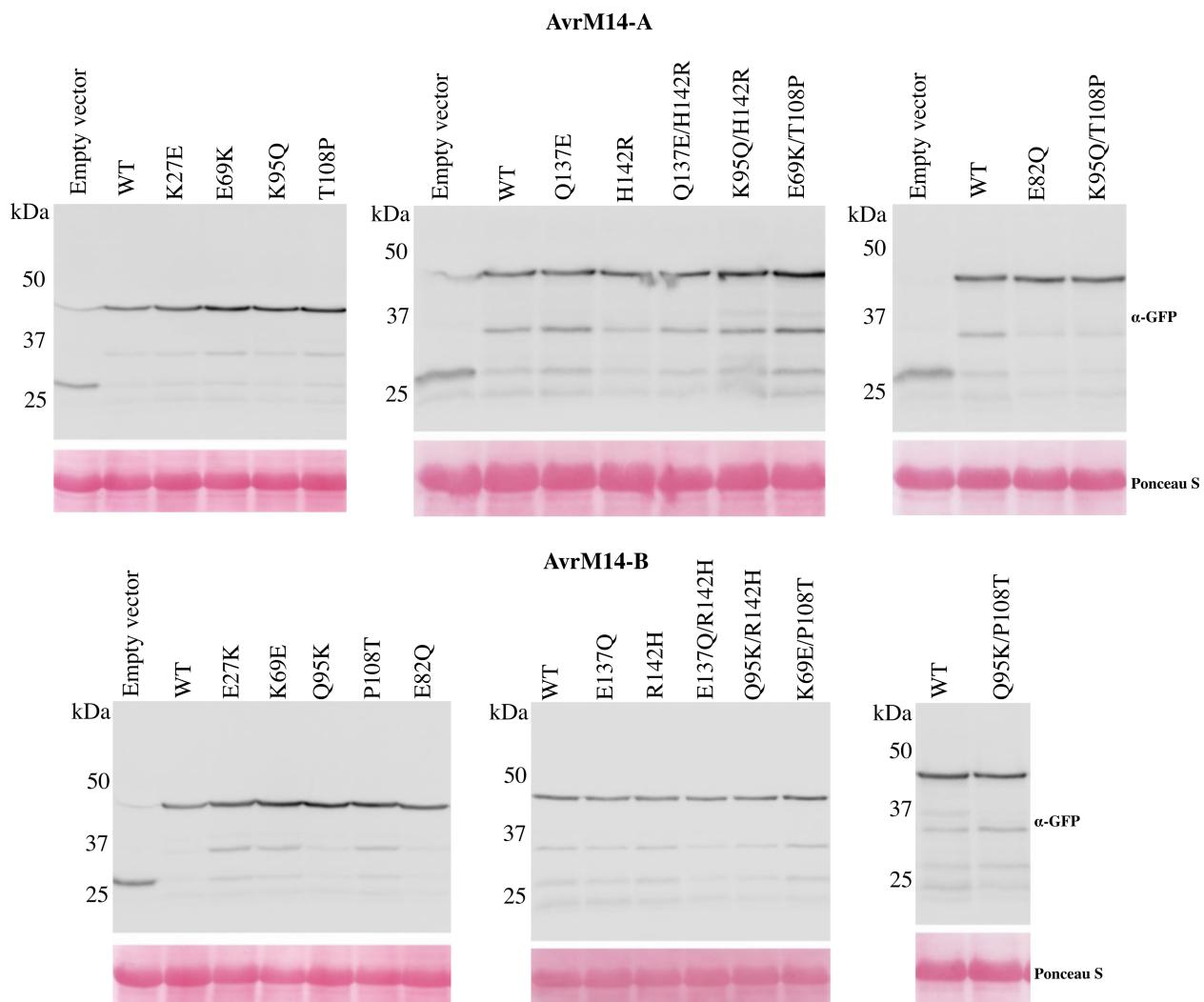


Fig. S3 The mutation of Nudix box glutamate E82 to glutamine does not alter the recognition of AvrM14-A or AvrM14-B by M1 when co-expressed in tobacco. (a) *Nicotiana tabacum* leaf sections were agroinfiltrated with a construct to express either M1, AvrM14-A, AvrM14-B, AvrM14-A^{E82Q} or AvrM14-B^{E82Q}. (b) *N. tabacum* leaf sections were co-agroinfiltrated with M1 and one of the AvrM14 constructs. Each leaf section is labelled with the name/s of the agroinfiltrated construct/s. Both leaves were imaged 6 days post-infiltration. The infiltrations were repeated 5 times with similar results obtained.

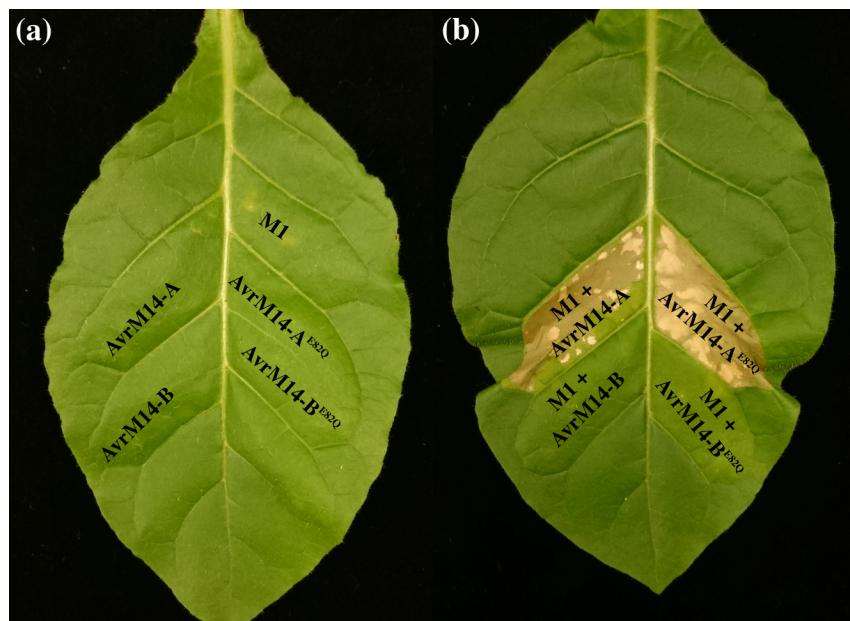


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(a) Size-exclusion chromatograms displaying the elution profile of each recombinant AvrM14 protein purified during this study. The green and purple shading indicate the peak representing monomeric and homodimeric protein, respectively. (b) Coomassie-stained SDS-PAGE analysis demonstrating the purity of the monomeric and homodimeric wild-type AvrM14 protein samples. (c) The crystal structures of monomeric AvrM14-A (left), AvrM14-B (middle) and homodimeric AvrM14-B (right) in cartoon form, demonstrating the similarities between the crystal packing dimers and the domain-swapped dimer. A zoomed-in view of the common dimeric interface observed in both AvrM14-B structures is shown in the middle, with the aromatic residues (F⁸⁹, F¹³⁸, and W¹⁴⁰) displayed as sticks.

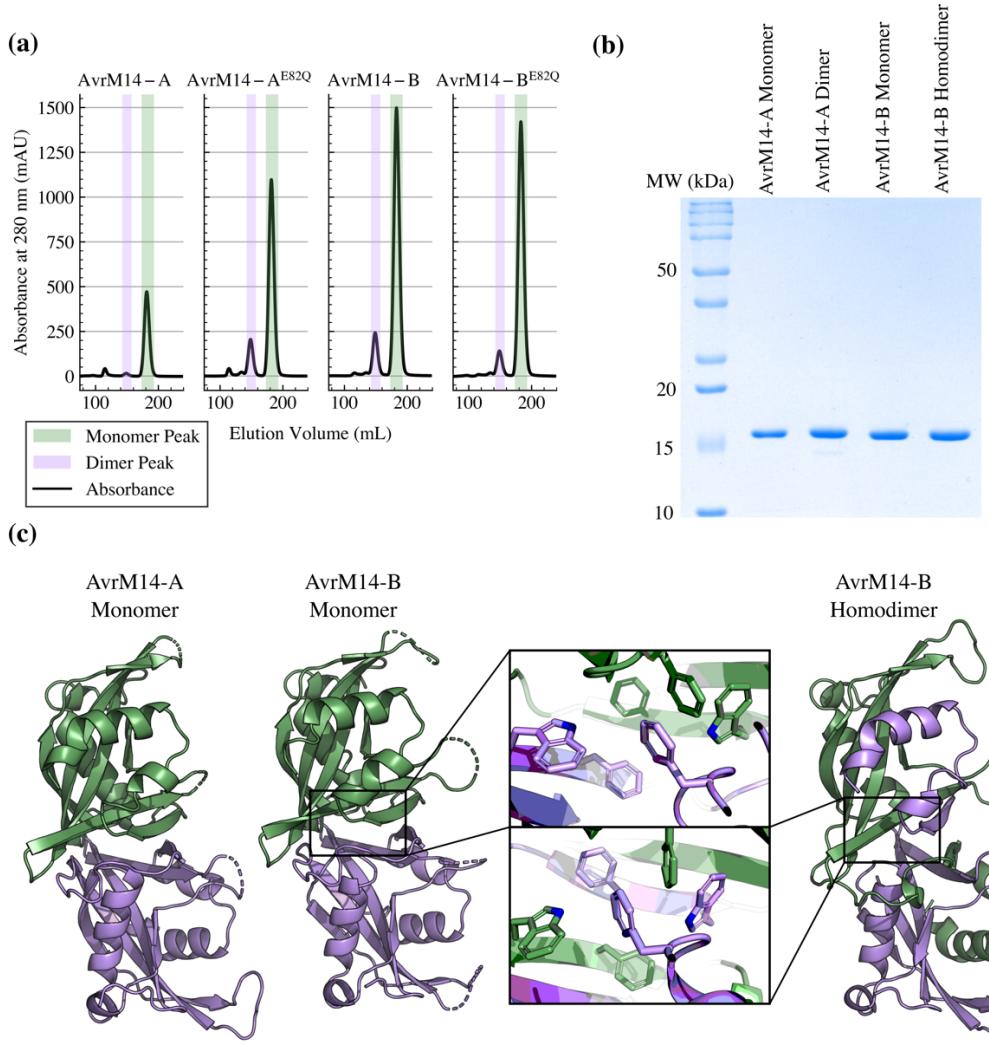


Fig. S5 The AvrM14-B crystal packing dimer (A, purple) compared to homodimeric *Homo sapiens* Nudt16 (PDB ID: 3MGM) (B, gold) with the Nudix helices coloured green.

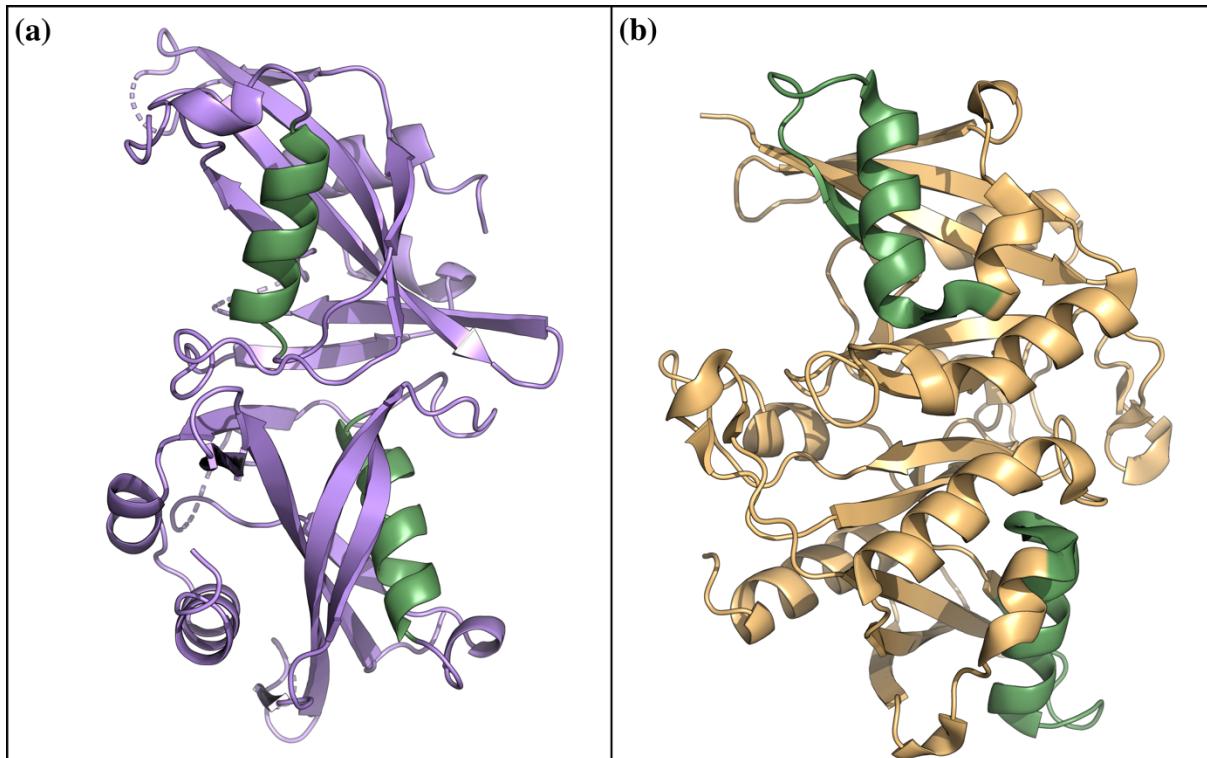


Fig. S6 Screening of common Nudix hydrolase substrates using monomeric and homodimeric AvrM14 protein. Recombinant AvrM14 monomeric and homodimeric proteins were incubated in a reaction buffer with potential substrate at 2 mM concentration for 30 minutes at 37 °C. Substrate hydrolysis was detected via the production of a blue-coloured phosphomolybdate complex that absorbs light with a wavelength of 820 nm; presented as mean absorbance \pm standard deviation ($n = 5$). A buffer only control without any Nudix hydrolase protein was used to blank the spectrophotometer before measurement. Recombinant AtNUDX7 and AtNUDX7^{E154Q} were used as positive and negative controls, respectively; the substrate hydrolysed is indicated at the top of each subplot.

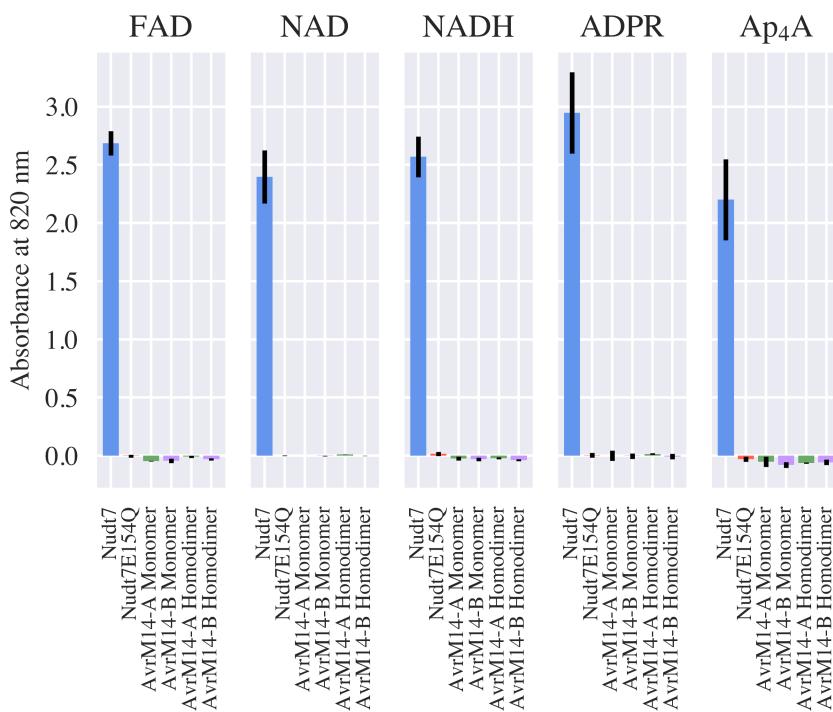
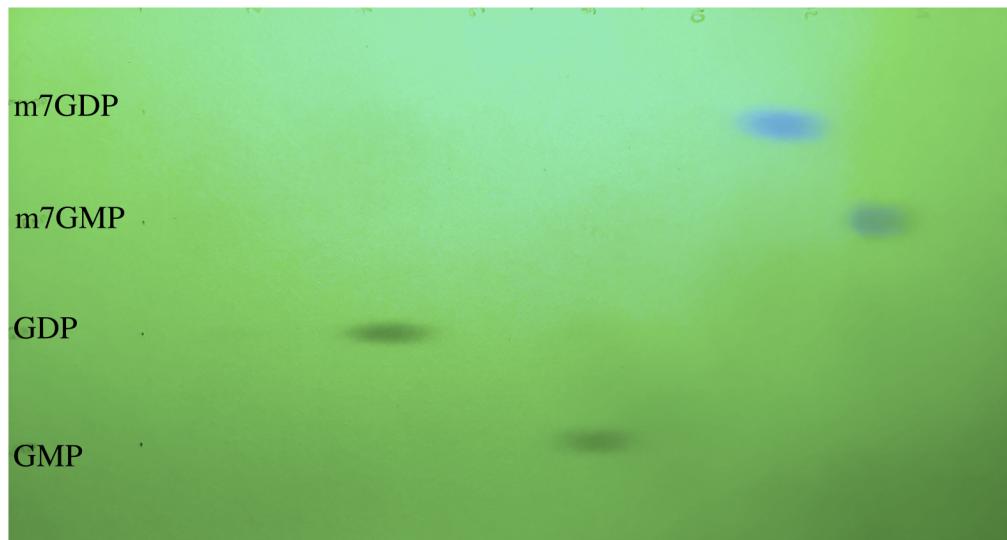


Fig. S7 The migration and Rf values for standard solutions of potential mRNA decapping products. (a) A thin-layer chromatography plate demonstrating the migration of standard solutions of m^7 GDP, m^7 GMP, GDP, and GMP. The plate was illuminated with 254 nm light to enable visualisation of the standards. (b) The migration of each standard was used to determine an Rf value. The mean Rf value from three replicates is listed.

(a)



(b)

Standard	Mean Rf Value
GMP	0.475
GDP	0.245
m^7 GMP	0.784
m^7 GDP	0.676

Fig. S8 Identifying stable multimeric AvrM14 protein complexes from *N. benthamiana* protein extracts. *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium* (GV3101) harbouring either an empty vector (pEG102) or pEG102 constructs encoding untagged AvrM14-A or AvrM14-B expressed under the 35S promoter from the cauliflower mosaic virus. At four days post-infiltration (dpi) leaf tissue was collected and total soluble protein was extracted. The protein solutions were loaded over a S75 10/300 analytical size-exclusion chromatography column. (a) The elution profile of the protein extract from plants transformed with AvrM14-A (red), AvrM14-B (gold), and the empty vector (blue). The peaks observed with purified recombinant AvrM14 monomeric and homodimeric under the same chromatography conditions are displayed in green and purple, respectively. (b) Immunoblot analysis with antibodies raised against AvrM14 was completed to determine the volume at which AvrM14 proteins were eluting from the column, from the plant tissue transformed with AvrM14-A (top), AvrM14-B (middle), and the empty vector (bottom). In all blots the lanes labelled with an 'A' contain 50 ng of recombinantly purified AvrM14-A and lanes labelled with a 'B' contain the plant protein extract before it was loaded onto the column. The numbers above the lanes indicate the elution volume of each fraction, as labelled on the chromatogram in (a).

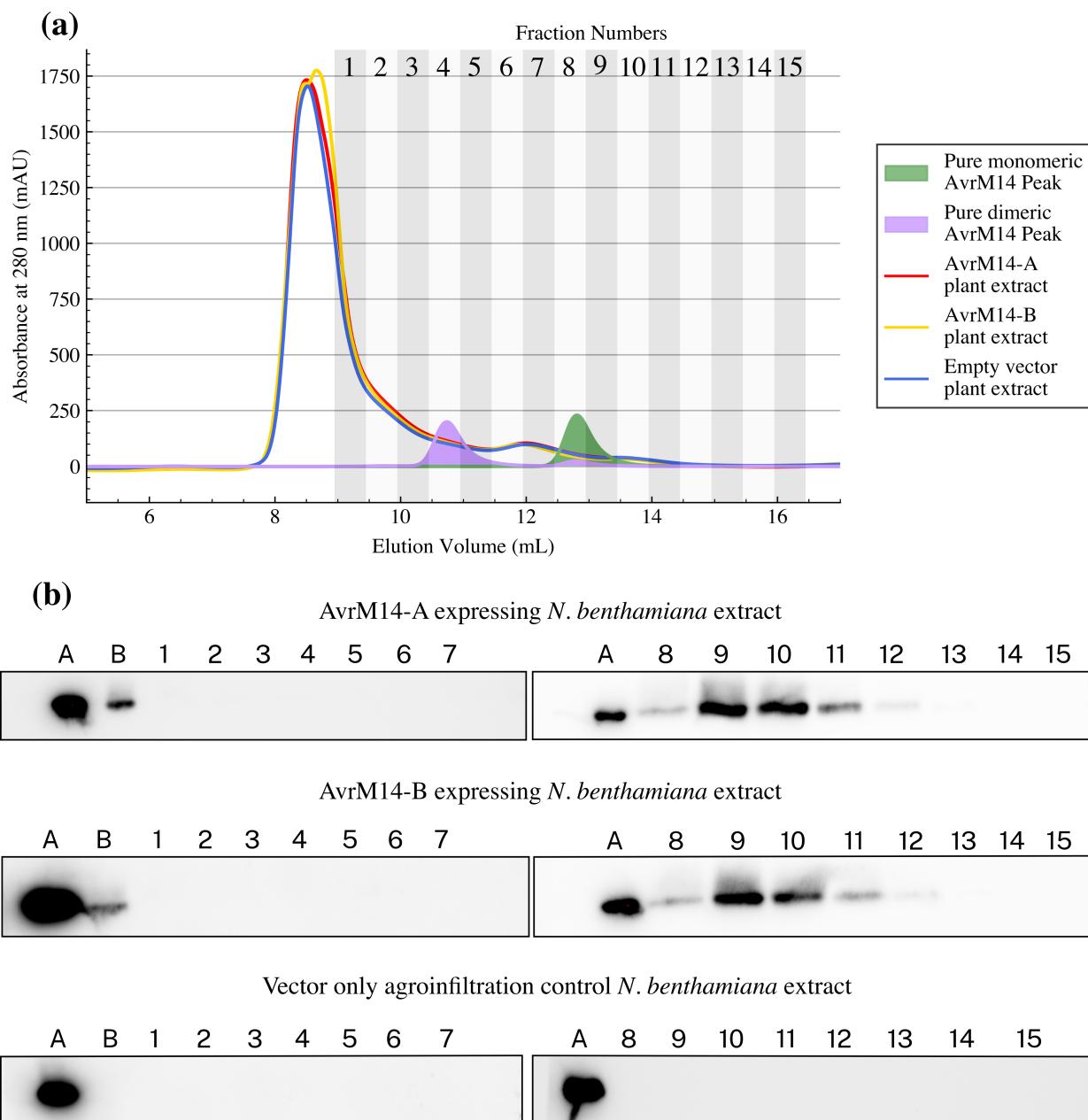


Fig. S9 Size-exclusion chromatograms displaying the elution profile of each of the AvrM14 homologues assessed in our study. For comparison, the elution volumes of monomeric and homodimeric AvrM14, using the same Superdex S75 26/600 column, are indicated by green and purple shading, respectively.

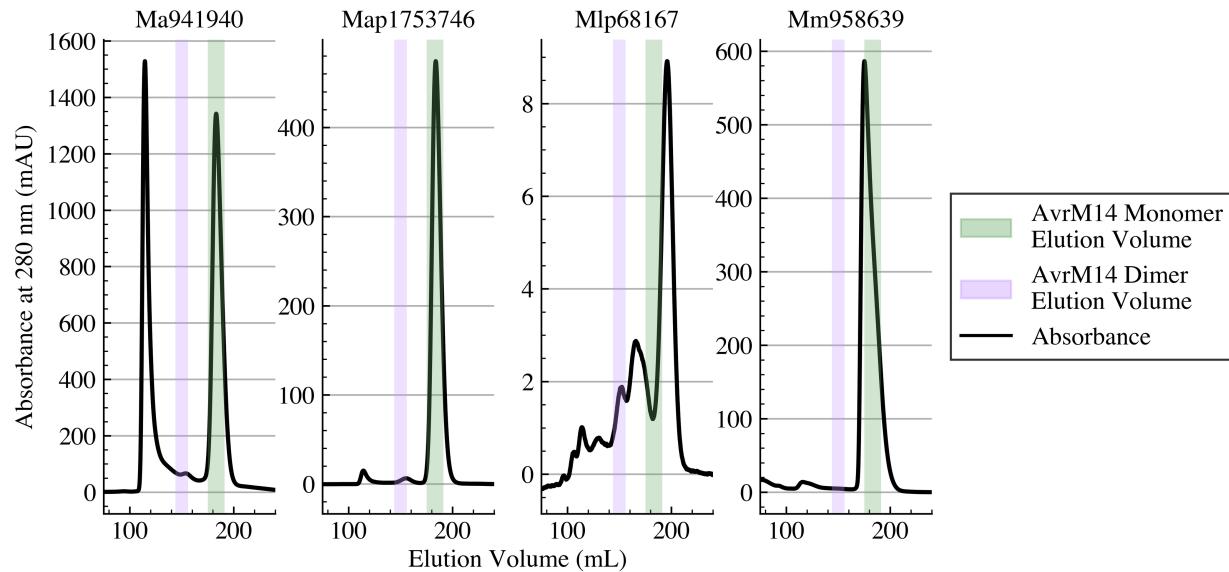


Fig. S10 The average expression level (\pm SD) in transcripts per million for AvrM14 genes in each of the RNA-Sequencing datasets.

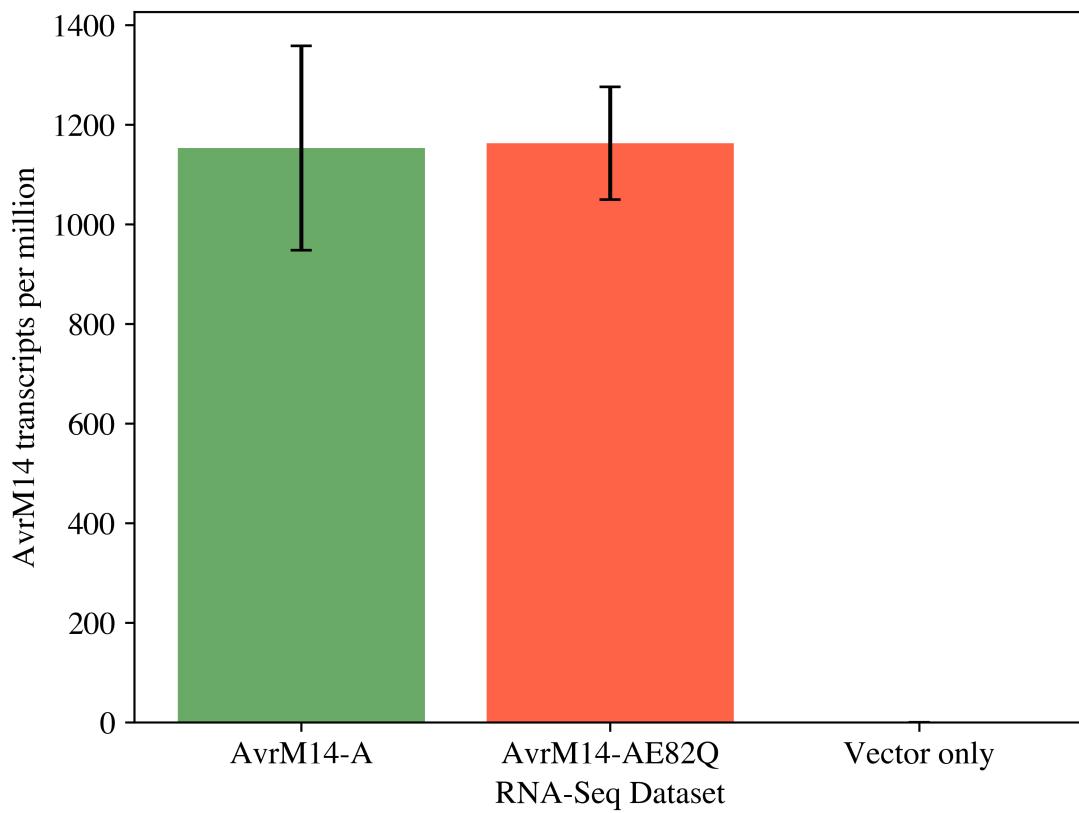


Fig. S11 Co-expression of AvrM14 wild-type and mutant proteins with M1 in tobacco. *Nicotiana tabacum* leaf sections were co-agroinfiltrated with a construct to express M1 and an AvrM14-A or AvrM14-B protein variant. The label on each leaf section indicates the AvrM14 protein being expressed as per the table below the images (e.g., the leaf section labelled with A-3 is expressing M1 and AvrM14-A^{E69K}, whereas the leaf section labelled with B-8 is expressing M1 and AvrM14-B^{E137Q/R142H}). The images shown are a representative result from between 4 to 15 replicates for each construct.



Label	Protein co-infiltrated with M1
A-1 / B-1	AvrM14-A / AvrM14-B
A-2 / B-2	AvrM14-A ^{K27E} / AvrM14-B ^{E27K}
A-3 / B-3	AvrM14-A ^{E69K} / AvrM14-B ^{K69E}
A-4 / B-4	AvrM14-A ^{K95Q} / AvrM14-B ^{Q95K}
A-5 / B-5	AvrM14-A ^{T108P} / AvrM14-B ^{P108T}
A-6 / B-6	AvrM14-A ^{Q137E} / AvrM14-B ^{E137Q}
A-7 / B-7	AvrM14-A ^{H142R} / AvrM14-B ^{R142H}
A-8 / B-8	AvrM14-A ^{Q137E/H142R} / AvrM14-B ^{E137Q/R142H}
A-9 / B-9	AvrM14-A ^{K95Q/H142R} / AvrM14-B ^{Q95K/R142H}
A-10 / B-10	AvrM14-A ^{E69K/T108P} / AvrM14-B ^{K69E/P108T}
A-11 / B-11	AvrM14-A ^{K95Q/T108P} / AvrM14-B ^{Q95K/P108T}

Fig. S12 Expression of wild-type and mutant AvrM14 proteins in *M1* and *M4* flax. Bison x M1 (Williston Brown) or M4 (Victory 'A') flax (*Linum usitatissimum*) lines were agroinfiltrated with a construct encoding either a wild-type (WT) or mutant AvrM14 protein. Multiple leaves from two or three independent plants were infiltrated with each construct. The agroinfiltrated leaves were photographed between 5 to 7 days post-infiltration to assess the cell-death response. Plants agroinfiltrated at the same time under identical conditions have been grouped together. Each group includes plants expressing WT AvrM14-A and AvrM14-B for comparison to the mutant constructs in the same group. The flax line being infiltrated is indicated at the top of each group; plants infiltrated with AvrM14-A variants are on the left and plants infiltrated with AvrM14-B variants are on the right. Whether the protein being expressed is WT or the identity of the residue/s mutated is listed on the far left of each group of images. For a summary of these results please see Supplementary Table 6.

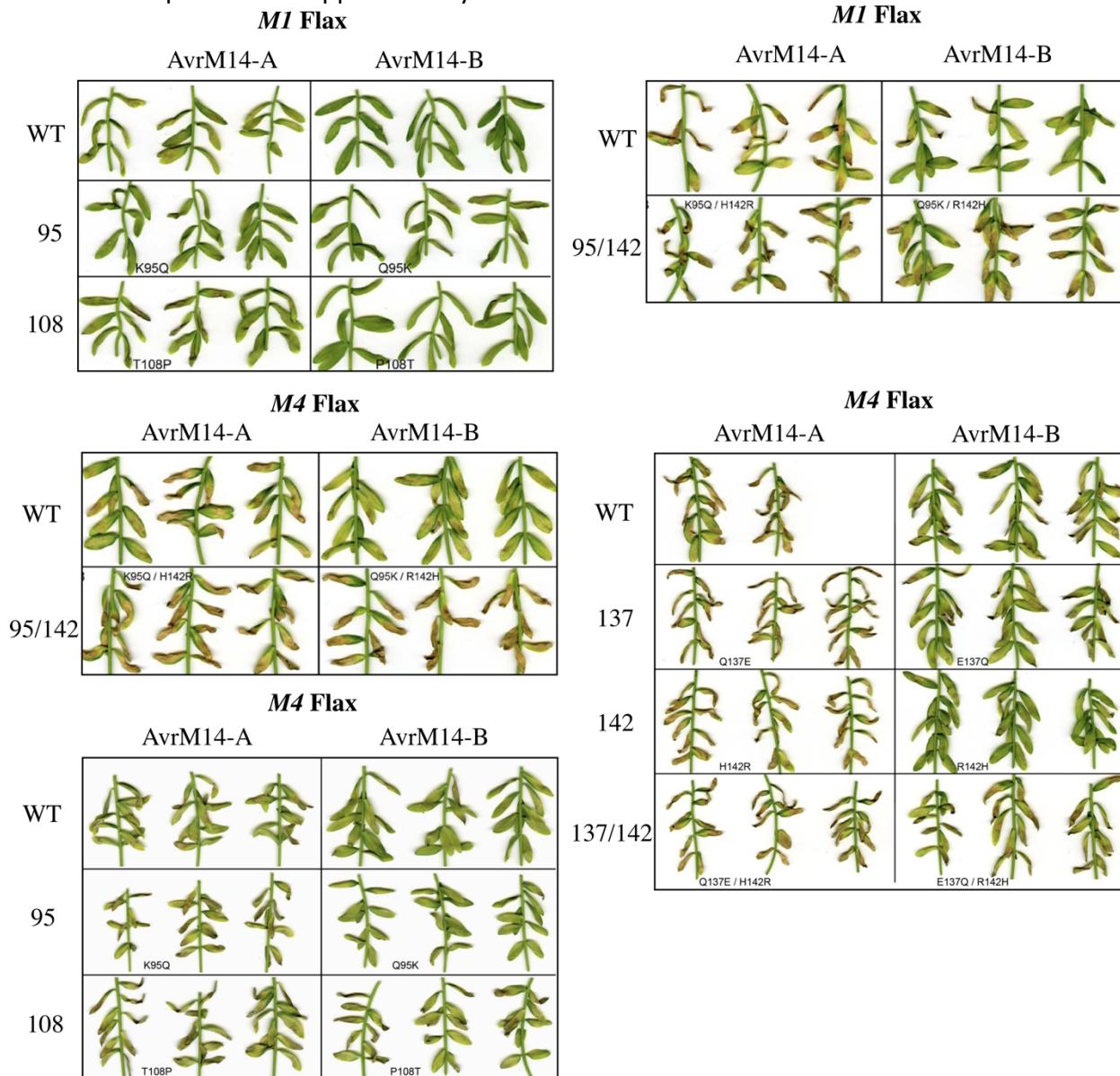


Table S1 DNA and protein sequences used in this study (see table S1.csv).

Table S2 Crystallography data collection and structure refinement statistics.

Protein	AvrM14-A		AvrM14-B Monomer	AvrM14-B Homodimer
	Bromide soak (SAD)	Native (MR)	Native (MR)	Native (MR)
Data collection				
Detector	ADSC-QUANTUM 315R	ADSC-QUANTUM 315R	Dectris Eiger X 16M	Dectris Eiger X 16M
Wavelength (Å)	0.9197	0.9537	0.9537	0.9537
Space group	P 21 21 21	P 21 21 21	P 61 2 2	P 41 21 2
a, b, c (Å)	38.645 63.241 167.195	37.645 166.842 62.911	80.059 80.059 293.949	79.663 79.663 201.317
α, β, γ (°)	90 90 90	90 90 90	90 90 120	90 90 90
Resolution (Å)	41.81 - 2.25 (2.33-2.25)	41.71 - 2.30 (2.38 - 2.30)	44.84 - 1.76 (1.82 - 1.76)	35.63 - 1.84 (1.90 - 1.84)
Total no. of reflections	383680 (32940)	108010 (10816)	1298084 (128096)	630813 (60186)
No. of unique reflections	20265 (1811)	18412 (1793)	56341 (5485)	57219 (5572)
Completeness (%)	99.9 (98.9)	99.8 (100)	99.9 (99.0)	99.5 (96.4)
Multiplicity	18.9 (18.2)	5.9 (6.0)	23.0 (23.4)	11.0 (10.8)
Anomalous completeness	99.8 (97.9)	-	-	-
Anomalous multiplicity	10.2 (9.6)	-	-	-
Mean I / s(I)	21.7 (4.3)	10.5 (2.4)	31.9 (4.2)	16.3 (0.8)
Rmerge (%)	0.125 (0.798)	0.160 (0.804)	0.068 (0.763)	0.089 (2.867)
Rmeas (%) ^c	0.128 (0.821)	0.175 (0.879)	0.070 (0.779)	0.0929 (3.008)
Rpim (%) ^d	0.029 (0.19)	0.070 (0.349)	0.015 (0.159)	0.028 (0.895)
CC _{1/2} ^b	0.99 (0.94)	0.99 (0.72)	1 (0.94)	1 (0.48)
Matthews coefficient (Å ³ Da ⁻¹) ^e	1.98	1.98	4.09	4.80
Phasing statistics determined by Crank2e				
No. of sites identified	24	-	-	-
Over all figure of merit (FOM)	0.286	-	-	-
FOM after density modification	0.649	-	-	-
Refinement				
Resolution range (Å)		41.71 - 2.30 (2.38 - 2.30)	44.84 - 1.76 (1.82 - 1.76)	35.63 - 1.84 (1.90 - 1.84)
R _{work} (%) ^g		18.3 (22.5)	17.7 (28.3)	19.0 (32.5)

$R_{\text{free}} (\%)^{\text{h}}$		25.15 (31.15)	20.14 (34.90)	22.14 (37.76)
No. of non-H atoms				
Total		3326	2438	2286
- Protein		3193	2162	2145
- Ligand		-	25	15
- Water		108	251	126
Average B -factor (\AA^2)		37.5	31.8	48.37
R.m.s.d. from ideal geometry				
Bond lengths (\AA)		0.008	0.009	0.010
Bond angles ($^{\circ}$)		0.982	0.990	0.960
Ramachandran plot, residues in (%) ⁱ				
Favoured regions		98.7	98.8	99.6
Additionally allowed regions		1.3	1.2	0.4
Outlier regions		0	0	0

Table S3 The substrates and secondary enzymes used in the phosphate sensing fluorophore-based hydrolysis assays with recombinant AvrM14-A.

Group	Substrate/s	Secondary enzyme
1	ADP-ribose GDP-glucose GDP-mannose	APase
2	CDP-choline CDP-glycerol TDP-glucose UDP-acetylglucosamine UDP-glucose	APase
3	Ap ₃ A Ap ₄ A Ap ₅ A Ap ₄ G	APase
4	Ap ₄ U Ap ₄ dT Ap ₅ G	APase
5	Gp ₂ G Gp ₃ G Gp ₅ G m ⁷ Gp ₃ C	APase
6	FAD Deamino-NAD ⁺ NAD ⁺ NADH	APase
7	N6-Me-ATP N1-Me-ATP dATP dADP ATP ADP 8-oxo-dATP 2'O-Me-ATP	PPase
8	N4-Me-dCTP CDP 5-OH-dCTP 5-MeOH-dCTP 5-Me-dCTP 2'O-Me-CTP	PPase
9	p ₄ G N1-Me-GTP dGTP dGDP GTP GDP 8-oxo-dGTP 8-oxo-GTP 3'-dGTP 2'O-Me-GTP	PPase
10	UTP UDP 5-Me-UTP 5-MeOH-UTP 2'O-Me-UTP	PPase
11	XTP TTP TDP ITP dITP	PPase
Ungrouped	ADP-glucose	APase
Ungrouped	GDP-fucose	APase
Ungrouped	UDP-galactose	APase
Ungrouped	UDP-glucuronic acid	APase
Ungrouped	m ⁷ Gp ₅ G	APase
Ungrouped	Ap ₆ A	APase
Ungrouped	CTP	PPase
Ungrouped	dCDP	PPase
Ungrouped	dUTP	PPase
Ungrouped	dCTP	PPase
Ungrouped	DHUTP	PPase

Table S4 The average read counts with standard deviations in TPM as determined by Salmon, the differential expression analysis output (log2fc and q-values) as determined by fishpond when comparing the AvrM14-A and vector only datasets, and the blast-2-go best hit for all transcripts (see table S4.csv).

Table S5 The average read counts with standard deviations in TPM as determined by Salmon, the differential expression analysis output (log2fc and q-values) as determined by fishpond when comparing the AvrM14-A and AvrM14-AE82Q datasets, and the blast-2-go best hit for all transcripts (see table S5.csv).

Table S6 Summary of the cell death results recorded during this study following the expression of various AvrM14 proteins in M1 flax (*Linum usitatissimum*), M4 flax, and tobacco (*Nicotiana tabacum*) co-expressing M1. The number represents the cell death response score following visual inspection of the leaves, whereby a higher score indicates increased cell death. Please see Fig 1, Fig 6, S3 Fig, S10 Fig, and S11 Fig for the original images. N/A values are listed if the protein/resistance protein combination was not tested in this study.

Mutation in AvrM14-A	M4 Flax	M1 Flax	M1 Tobacco	Mutation in AvrM14-B	M4 Flax	M1 Flax	M1 Tobacco
Wild-Type	3	2	2	Wild-Type	0	0	0
K95Q	3	0 - 1	0	Q95K	0	0 - 1	0 - 1
T108P	3	2	0 - 1	P108T	1	0	0
K95Q/T108P	0	0	0	Q95K/P108T	3	3	0 - 1
Q137E	3	N/A	2	E137Q	0	N/A	0
H142R	3	N/A	2	R142H	0	N/A	0 - 1
Q137E/H142R	3	N/A	1	E137Q/R142H	0 - 1	N/A	0 - 1
K95Q/H142R	4	3	0	Q95K/R142H	3	2	0 - 1
K27E	N/A	N/A	2	E27K	N/A	N/A	0
E69K	N/A	N/A	2	K69E	N/A	N/A	0
E69K/T108P	N/A	N/A	2	K69E/P108T	N/A	N/A	0
E82Q	4	3	2	E82Q	0	0	0

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Chapter 3 A family of fungal effectors induce plant phosphate starvation responses by hydrolysing inositol pyrophosphates

In chapter 2, I established that AvrM14 and sequence-related effectors from the *Melampsora* genus are Nudix hydrolase enzymes. The effectors can remove the protective 5' cap from *in vitro* transcribed mRNA; notably, AvrM14 demonstrates remarkable substrate specificity, only hydrolysing capped mRNA and a molecule analogous to the 5' cap structure. I also report that AvrM14 hydrolase activity suppresses immune responses in both *N. benthamiana* and flax leaves. Overall, the results support a model whereby the *Melampsora* Nudix hydrolase effectors decap plant mRNA to suppress immune responses and ultimately promote the infection process. Following the AvrM14 study, I aimed to characterise the second identified family of predicted fungal Nudix hydrolase effectors.

Genome sequencing of *M. oryzae* in 2005 identified two identical copies of a putative Nudix effector called *MoNudix* (Dean *et al.*, 2005). Subsequent studies identified similar predicted Nudix effectors with approximately 40% amino acid sequence identity in *C. lentic*, *C. higginsianum*, *C. graminicola*, and *C. fructicola* (Bhaduria *et al.*, 2013, Crouch *et al.*, 2014). This chapter investigates the Nudix effectors from *Magnaporthe* and *Colletotrichum* genera. In this chapter, I use a combination of structural biology, enzymatic assays, and *in planta* experiments to characterise the *Magnaporthe* and *Colletotrichum* Nudix effectors. After a detailed analysis of the Nudix effectors in heterologous systems, we established a collaboration with Dr Ely Oliveira-Garcia at Louisiana State University to conduct experiments in the native *M. oryzae* system. Dr Oliveira-Garcia and his student Chenie Zamora used RNA interference to probe *MoNudix*'s contribution to rice blast disease symptoms, and live-cell imaging to assess *MoNudix*'s localisation during rice infection. Our collective research on the *Magnaporthe* and *Colletotrichum* Nudix effectors culminated in the preparation of a manuscript, completed in August 2023, which is presented in this chapter. For this manuscript, I wrote the initial draft and created all the figures, except Figure 4. See the statement of contribution for more details.

I presented my research on *MoNudix* at the International Society for Plant-Microbe Interactions 2023 Congress, Providence, Rhode Island in August. During my poster presentation I was approached by Florencia Casanova and Louisa Wirtz from Prof. Ulrich Schaffrath's group at

RWTH Aachen University, Germany. Florencia and Louisa informed me that the Schaffrath lab was involved in research concerning *MoNudix*. After the meeting we contacted Prof. Schaffrath and his research team, sharing our respective data sets. The Schaffrath lab had an unpublished manuscript, close to submission, which demonstrates that knocking out *MoNudix* in *M. oryzae* significantly reduces disease symptoms on multiple cultivars of rice and barley. They had also completed complementation experiments demonstrating that *MoNudix*'s hydrolase activity is essential for promoting disease. Collectively, our research was highly complementary, and we therefore decided to combine our manuscripts into a single manuscript. I wrote the initial draft of the combined manuscript and assembled all figures, except supplemental figure 1. The combined manuscript has been submitted to a journal and made available on the preprint server bioRxiv (insertDOIhere). It can also be found as appendix 1 in this thesis.

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Title

A family of fungal effectors induce plant phosphate starvation responses by hydrolysing inositol pyrophosphates

Authors

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Summary (<150 words)

Plant phosphate starvation response transcription factors (PHRs) play an essential role in maintaining phosphate homeostasis. Crucial links between plant PHRs and interactions with beneficial microbes, such as phosphate-providing arbuscular mycorrhizal fungi, are well established. In this study, we have identified a conserved effector family in pathogenic *Magnaporthe* and *Colletotrichum* fungi capable of activating PHRs *in planta*. We demonstrate that the effectors hydrolyse inositol pyrophosphates (PP-InsPs), which are used by plants to signal changes in phosphate availability and regulate PHR activity. In *M. oryzae*, the reduction of one PP-InsP hydrolase effector through RNA interference (RNAi) significantly diminishes virulence on rice, while simultaneously promoting reactive oxygen species (ROS) accumulation around the infection site. Altogether, our research elucidates a molecular mechanism whereby various pathogenic fungi directly target a conserved signalling molecule involved in phosphate homeostasis to disrupt plant defence, activate PHRs, and ultimately enhance disease progression.

Keywords

Phosphate starvation, Plant immunity, virulence effector, Nudix hydrolase, *Magnaporthe oryzae*

Introduction

Inositol pyrophosphates (PP-InsPs) are small molecules composed of a fully phosphorylated inositol ring with either one or two pyrophosphate moieties. PP-InsPs have the most concentrated array of phosphates in nature, resulting in a dense electronegative charge.¹ The intracellular concentration of PP-InsPs is linked to the availability of inorganic phosphate (Pi),^{2,3} and plants sense PP-InsP levels to control phosphate starvation responses (PSRs).²⁻⁶ Within eukaryotic cells, the electronegative PP-InsPs bind to basic surface clusters on SPX protein domains (named after yeast SYG1, Pho81, and human XPR1) to facilitate Pi sensing.² SPX domains are found in various proteins involved in eukaryotic phosphate homoeostasis and as single-domain proteins.⁷⁻¹⁴ In plants, single-domain SPX proteins act as PP-InsP-dependent repressors of the phosphate starvation response transcription factors (PHRs);^{4,15} PHRs complete extensive transcriptional reprogramming during phosphate starvation.¹⁶⁻¹⁸ When phosphate is abundant, PHRs are held in an inactive state by PP-InsP-bound SPX proteins.^{4,11,13-15} During phosphate starvation, PP-InsP levels decrease and PHRs are released by SPX proteins, resulting in the transcription of target genes. Notably, these target genes typically contain PHR binding sequences (P1BSs) (GnATATnC) within their promoter region.^{16,17}

Symbiosis with arbuscular mycorrhizal fungi (AMF) enables c. 80% of land plants to access more phosphate and other mineral nutrients from the environment in exchange for carbon.¹⁹ In soil with high levels of accessible phosphate, plants suppress AMF symbiosis, potentially to conserve carbon.²⁰⁻²⁸ Likewise, interactions between *Arabidopsis thaliana* and phosphate-providing endophyte *Colletotrichum tofieldiae* are regulated by plant phosphate status.²⁹⁻³¹ Recent research indicates that the regulation of AMF symbiosis is primarily mediated by PHRs.^{32,33} PHR transcriptional activity also represses certain plant defence responses, possibly to help facilitate interactions with beneficial microbes and/or prevent defence-mediated suppression of phosphate transport.³⁴⁻³⁶ One mechanism by which PHRs can influence plant immune function and promote root colonisation by phosphate providing microbiota, is by activating the expression of rapid alkalinisation factor (RALF) genes that possess the P1BS element within their promoter.³⁵ The receptor kinase FERONIA senses RALF peptides, which results in reduced assembly of the immune receptor complex composed of flagellin sensing 2 (FLS2) and BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1).³⁷ Consequently, under phosphate-starved conditions, *A. thaliana* plants demonstrate compromised immune signalling pathways.³⁵

To facilitate infection, pathogenic microbes can secrete proteins known as effectors to manipulate the host plant. Effectors often suppress plant immune responses. Increasing evidence suggests that plant pathogens may specifically target PSRs to promote disease.³⁸ SAP11 is an effector from a parasitic phytoplasma capable of inducing PSRs and suppressing defense in transgenic *A. thaliana*,³⁹ potentially via PHR activation.³⁸ Likewise, infection with *Candidatus liberibacter asiaticus* bacteria induces PSR symptoms in the host *Citrus sinensis*.⁴⁰ Another effector, XopH, from *Xanthomonas campestris* pv. *Vesicatoria*, reduces plant PP-InsP levels by dephosphorylating inositol hexakisphosphate (InsP6), although it remains unknown if XopH induces PSRs.⁴¹ In addition to the suppression of plant immunity, pathogens may benefit from the increase in bioavailable intracellular phosphate resulting from the activation of PSRs.³⁸ For example, excessive phosphate supply renders rice plants more susceptible to *Magnaporthe oryzae*.⁴²

Nucleoside diphosphate linked to moiety-X (Nudix) hydrolase domains have been predicted in effectors from fungal, oomycete and bacterial plant pathogens.⁴³⁻⁴⁸ Nudix hydrolases share a consensus sequence called the Nudix box (GX₅EX₇REUXEEGU, where U represents a hydrophobic amino acid and X is any amino acid). Typically, Nudix hydrolases catalyse the hydrolysis of nucleoside diphosphate-containing compounds.⁴⁹ However, a subset of Nudix hydrolases can also hydrolyse diphosphate bonds in compounds without a nucleoside, including PP-InsPs.⁵⁰⁻⁵³ In this study, we identify a conserved group of Nudix hydrolase effectors from economically important pathogenic fungi, including *Magnaporthe oryzae*, the causative agent of rice blast disease, and multiple *Colletotrichum* species responsible for anthracnose disease on a wide variety of plants. Through a combination of experimental and computational structural biology, *in vitro* enzymatic assays, and *in planta* analysis, we establish that Nudix hydrolase effectors from *M. oryzae* and *Colletotrichum spp.* possess a conserved basic PP-InsP binding site, exhibit PP-InsP hydrolysis activity, and employ their hydrolase activity to induce PSRs and suppress immune-activated reactive oxygen species (ROS) production in plants. Additionally, our results indicate that an *M. oryzae* PP-InsP hydrolase effector is secreted into the cytoplasm of host rice cells during infection, and we demonstrate that this effector suppresses host immune responses and significantly contributes to the virulence of *M. oryzae*.

Results

Putative Nudix effectors are widespread in *Magnaporthe* and *Colletotrichum* pathogenic fungi

Recently, we demonstrated that Nudix effectors from pathogenic fungi in the *Melampsora* genus exhibit mRNA decapping activity.⁴⁶ Within fungi, the plant pathogens *C. lenthis* (previously *C. truncatum*), *M. oryzae*, and *C. higginsianum* also have predicted Nudix effectors.^{44,54,55} While the biochemical activity of these remains unknown, studies suggest they play a role in pathogen virulence. In *Colletotrichum*, Bhaduria *et al.*, (2013) suggested that *CtNudix*, a Nudix effector from *C. lenthis*, is important for the transition to necrotrophic growth,⁴⁴ and multiple putative Nudix effectors are specifically and highly upregulated during the biotrophic growth phase of *C. higginsianum*.⁵⁵ More recently, Yan *et al.*, (2023) reported that two identical Nudix effector genes from *M. oryzae* (MGG_14156 and MGG_14344, referred to here as *MoNudix*) were consistently among the top ten upregulated predicted effectors at 48 to 96 hours post inoculation (hpi) in three independent infection experiments involving two different rice cultivars.⁵⁴

Sequence analysis indicates that the *Magnaporthe* and *Colletotrichum* Nudix effectors are more closely related to each other than to the mRNA decapping *Melampsora* effectors, and resemble a distinct effector family (Figure 1A). We investigated the prevalence of the *Colletotrichum* and *Magnaporthe* Nudix hydrolase effector family in pathogenic and non-pathogenic fungi. Previous studies have reported sequence-related Nudix effectors in *C. higginsianum*, *C. graminicola*, *C. fructicola*, *C. orbiculare*, *C. lenthis*, and *M. oryzae*.^{44,56} To further explore the Nudix effector family, we searched NCBI databases (see ‘Methods’ for detailed information) to identify similar putative Nudix effectors. Our search recovered Nudix effectors (with a Nudix domain and a predicted signal peptide) across *Magnaporthe*, *Colletotrichum* and *Ceratocystis* genera (Figure 1A, Table S1). Notably, all predicted Nudix effector genes belong to pathogenic species, while no predicted Nudix effectors were identified in *C. tofieldiae*, a closely related genome-sequenced endophytic species.³⁰ Collectively, the fungi that possess predicted Nudix effectors infect a diverse range of monocot and dicot plant species (Table S1), suggesting that any shared target/s of the Nudix effectors may be widely conserved in land plants.

Magnaporthe and *Colletotrichum* Nudix effectors inhibit the ROS burst and specifically hydrolyse PP-InsPs

Previously characterised Nudix hydrolase effectors from plant pathogens, including Avr3b from *Phytophthora sojae*, RipN from *Ralstonia solanacearum*, and AvrM14 from *Melampsora lini*, can use hydrolase activity to inhibit plant immune signalling.^{43,45,46} To investigate whether Nudix effectors from *Magnaporthe* and *Colletotrichum* can also disrupt plant immune signalling through hydrolase activity, we utilised Agrobacterium-mediated transformation of *N. benthamiana* to transiently express *MoNudix*, and a Nudix effector from *C. higginsianum* (*ChNudix*), as well as mutant proteins where a conserved Nudix box glutamate (E) typically essential for hydrolase activity, was substituted to glutamine (Q) (*MoNudix*^{E79Q} and *ChNudix*^{E78Q}). Our results clearly demonstrate that the expression of *MoNudix* and *ChNudix*, but not the catalytic mutants (E-to-Q), significantly impairs the production of ROS in *N. benthamiana* leaf tissue upon exposure to either flg-22 or chitin (Figure 1B). Western blot analysis of soluble protein extracts from *N. benthamiana* confirms the expression and accumulation of all effector proteins (Figure S1).

To understand how the Nudix hydrolase activity of the *Magnaporthe* and *Colletotrichum* effectors interfere with flg-22 and chitin responses, we sought to identify the substrate/s hydrolysed by the effectors. For initial substrate screening, we utilised recombinant *MoNudix* effector protein produced in *E. coli* and purified to homogeneity (Figure S2). We screened multiple common substrates of Nudix hydrolases, including those identified as substrates (ADP-ribose, NADH, and mRNA caps) of the previously characterised Nudix effectors (Avr3b, RipN, and AvrM14).^{43,45,46} *MoNudix* did not efficiently hydrolyse any Nudix hydrolase substrates tested (Figure S3A and S3B), suggesting that the *Magnaporthe* and *Colletotrichum* Nudix effector family are functionally distinct from previous studied Nudix hydrolase effectors. Interestingly, the *Magnaporthe*, *Colletotrichum*, and *Ceratocystis* Nudix effectors share a conserved glycine at position +1 in the Nudix box (Figure S4A), which has been demonstrated to be important for the enzymatic activity of a human PP-InsP hydrolase, *HsDIPP1*.^{57,58} Furthermore, we utilised AlphaFold⁵⁹ to model the protein structures of the Nudix effector family, and the predicted structures exhibit similarity to *HsDIPP1* (Figure S4B). The prediction of structural similarity between *MoNudix* and *HsDIPP1* is consistent with recent publications that reported AlphaFold models for *M. oryzae* effectors.^{54,60}

To determine if *MoNudix* can hydrolyse PP-InsPs *in vitro*, we first synthesised 5-PP-InsP₅ using inositol hexakisphosphate (InsP₆) and an *Entamoeba histolytica* InsP₆ kinase (*EhIP6KA*).⁶¹ 5-PP-InsP₅ is one of the PP-InsPs produced in plants, and is involved in the

regulation of the PSR in a PHR-dependent manner.⁶ We then incubated the 5-PP-InsP₅ with purified recombinant *MoNudix* protein and utilised polyacrylamide gel electrophoresis to separate the inositol polyphosphate species. This assay demonstrates that *MoNudix* converts 5-PP-InsP₅ into InsP₆ (Figure 1C). Importantly, like ROS suppression, the enzymatic activity requires the Nudix box, as the glutamate to glutamine substitution (*MoNudix*^{E79Q}) abolishes 5-PP-InsP₅ hydrolysis (Figure 1C). To establish if this enzymatic activity is conserved in the extended Nudix effector family, we also evaluated whether *ChNudix* and a second *M. oryzae* effector (*MoNudix2*) (purified to homogeneity (Figure S2)), could hydrolyse 5-PP-InsP₅. Like *MoNudix*, *ChNudix* and *MoNudix2* both effectively hydrolyse 5-PP-InsP₅ *in vitro* using their Nudix box (Figure 1D and 1E). In contrast to the *Magnaporthe* and *Colletotrichum* Nudix effectors, the *M. lini* Nudix effector AvrM14-A cannot hydrolyse 5-PP-InsP₅ in our assay system, and neither can *AtNudx7*, a Nudix hydrolase with broad substrate specificity that negatively regulates plant immunity in *A. thaliana*⁶² (Figure S3C). Overall, our *in vitro* enzymatic assays demonstrate that the *Magnaporthe* and *Colletotrichum* Nudix effectors are PP-InsP hydrolases (Figure 1F).

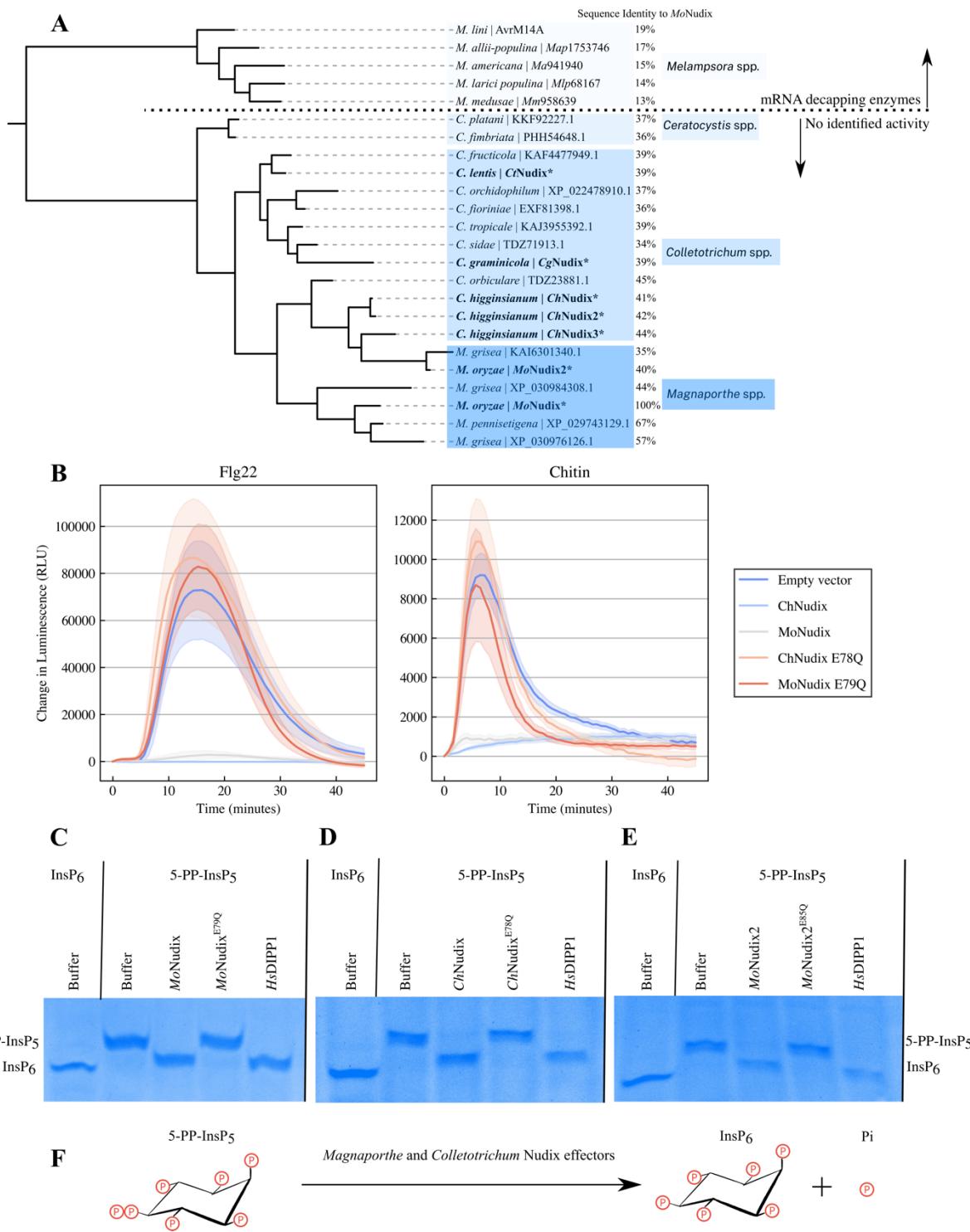


Figure 1: Nudix effectors from *Magnaporthe* and *Colletotrichum* pathogenic fungi hydrolyse inositol pyrophosphates and their hydrolase activity suppresses the flg-22- and chitin-triggered ROS burst in *Nicotiana benthamiana*. (A) A maximum-likelihood phylogeny estimated with PhyML⁶³ and visualised with iTOL,⁶⁴ using the protein sequences of select Nudix hydrolase effectors from pathogenic fungi. Each leaf is labelled with the species and protein names, those in bold and indicated with an * are analysed in this study (amino acid sequences in Table S1). The percentage next to each label indicates amino-acid sequence identity to *MoNudix*. The black dotted line separates effectors with no identified enzymatic activity from those previously characterised as mRNA decapping enzymes.⁴⁶ (B) *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* (GV3101) harbouring either an empty vector or constructs encoding *MoNudix*, *ChNudix*, *MoNudix*^{E79Q}, or *ChNudix*^{E78Q} proteins with 3xHA N-terminal tags expressed under the 35 S promoter from the cauliflower mosaic virus. At 3 days post-infiltration (dpi), leaf tissue was exposed to 100 nM flg-22 (left) or 5 µg ml⁻¹ chitin (right) and ROS production was recorded as relative luminescence units over time. Results are mean (solid line) ± SEM (shaded area) where n = 8. (C), (D), and (E) Purified recombinant protein (*MoNudix*, *MoNudix*^{E79Q}, *HsDIPP1*, *ChNudix*, *ChNudix*^{E78Q}, *MoNudix2*, and *MoNudix2*^{E85Q}) at a concentration of 5 µM or protein storage buffer was incubated with either inositol hexakisphosphate (InsP₆) or an inositol pyrophosphate (5-PP-InsP₅) (indicated along the top of the gel) for 60 minutes at 37 °C. The reaction products were separated using a polyacrylamide gel and visualised by staining with toluidine blue. The downwards shift of the band in the *MoNudix*, *HsDIPP1*, *ChNudix*, and *MoNudix2* lanes when compared to the other 5-PP-InsP₅ treatments, indicates that these proteins convert 5-PP-InsP₅ to InsP₆ (as depicted in (F)).

A conserved, positively charged, binding site in the *Magnaporthe* and *Colletotrichum* Nudix effectors is required for 5-PP-InsP₅ hydrolysis

To enhance our understanding of *MoNudix* as a PP-InsP hydrolase, we sought to determine the protein's structure as a ligand bound complex. Access to sufficient PP-InsP for co-crystallisation was not possible. We instead performed co-crystallisation of *MoNudix* in saturating concentrations of InsP₆ (the product of PP-InsP hydrolysis), which has a disassociation constant (Kd) of 6.02 ± 1.36 mM with *MoNudix* (Figure 2A), calculated using micro-scale thermophoresis (MST).

The crystal structure of *MoNudix*, determined at a resolution of 1.6 Å (PDB ID: 8SXS), revealed a characteristic Nudix fold that shares a highly similar overall structure (TM score = 0.77) with *HsDIPP1*,⁵⁸ despite low sequence identity (c. 25%) (Figure 2B). While InsP₆ was required for crystallisation of *MoNudix*, the unambiguous placement of InsP₆ within the electron density maps was not possible, likely due to ligand occupancy. To interrogate the PP-InsP binding site and identify amino acids involved in substrate binding, we superimposed the structure of *MoNudix* with the 5-PP-InsP₅ bound *HsDIPP1* structure (PDB ID: 6wo7)⁵⁸ (Figure 2C). Analogous to *HsDIPP1*, the predicted PP-InsP binding site in *MoNudix* is characterised by basic amino acids (lysines and arginines), creating a positively charged putative binding cleft located adjacent to the negatively charged Nudix active site (Figure 2C and 2D). We targeted two lysine amino acids (K53 and K142), predicted to be involved in PP-InsP interactions, and substituted them with glutamate (*MoNudix*^{KKEE}). Using MST, we demonstrate that purified *MoNudix*^{KKEE} (Figure S2) exhibits a 70-fold reduction in InsP₆ binding affinity (Figure 2A and 2E). Moreover, *MoNudix*^{KKEE} is unable to hydrolyse 5-PP-InsP₅ *in vitro* (Figure 2F).

To identify conserved structural elements across the *Magnaporthe*, *Colletotrichum* and *Ceratocystis* Nudix effector family, we employed ConSurf⁶⁵ to independently identify homologous sequences and map sequence conservation onto the *MoNudix* protein surface (Figure 2G). The proposed 5-PP-InsP₅ binding site, including the basic amino acids (required for substrate binding) and the Nudix box, exhibits high conservation (Figure 2G, Figure S4A). The conservation of the PP-InsP binding site, and enzymatic activity in *ChNudix* and *MoNudix2* (Figure 1C), suggests that PP-InsP hydrolase activity is likely conserved across the sequence-related *Magnaporthe*, *Colletotrichum*, and *Ceratocystis* Nudix effector family.

The crystal structure also reveals a single disulphide bond in the *MoNudix* protein, which serves to anchor the Nudix helix to a β -sheet, potentially providing additional structural stability (Figure S4C). Analogous disulphide bonded cysteines have not been reported previously in Nudix hydrolase enzymes and are a conserved and unique feature of this fungal PP-InsP effector family (Figure S4A).

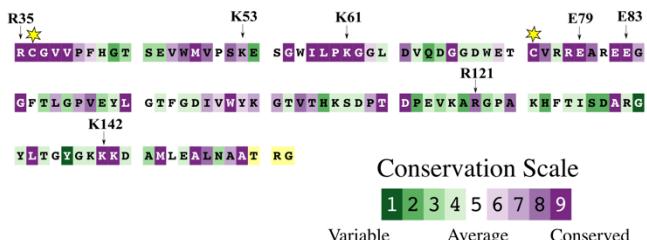
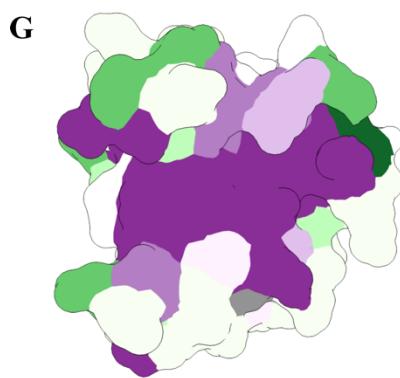
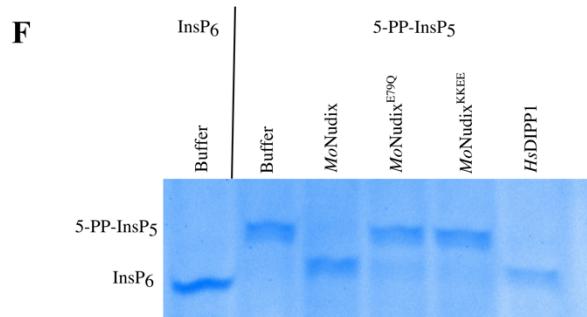
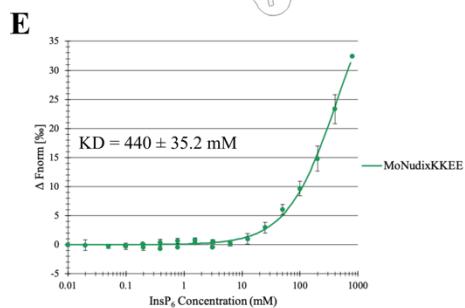
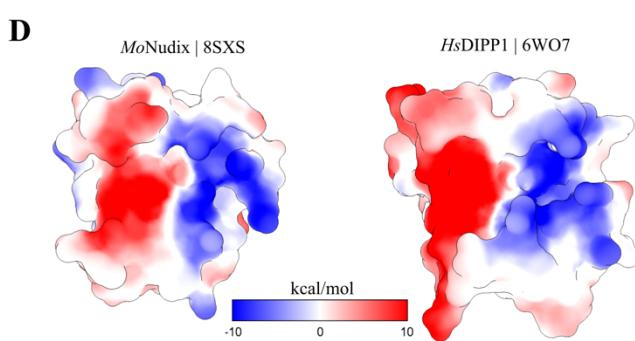
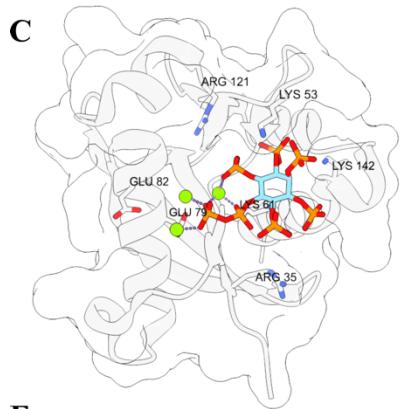
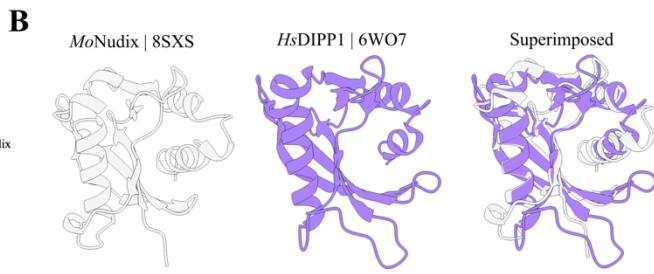
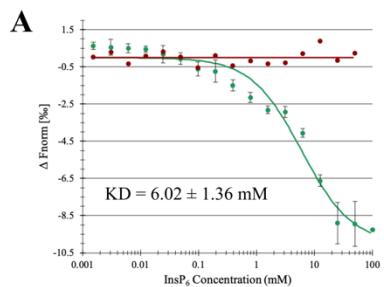


Figure 2: The crystal structure of *MoNudix* highlights a conserved, highly charged, binding site involved in inositol polyphosphate binding and hydrolysis. (A) and (E) Normalized microscale thermophoresis (MST) binding curves of *MoNudix* plus a protein storage buffer control (A) and *MoNudix*^{KKEE} (E) in the presence of InsP₆. The binding curve yields a Kd of 6.02 ± 1.32 mM for *MoNudix* and a Kd of 440 ± 35.2 mM for *MoNudix*^{KKEE}. Protein concentrations were kept constant while the InsP₆ concentration varied. (B) Ribbon diagrams of *MoNudix* (PDB ID: 8SXS) (grey) and *HsDIPP1* (PDB ID: 6WO7)⁵⁸ (purple) crystal structures superimposed to demonstrate their striking structural similarity (TM score = 0.77) despite low levels of sequence identity (25%). (C) A ribbon diagram of *MoNudix* with an overlaid transparent surface model with Mg²⁺ and 5-PP-InsP₅ docked into the structure via structural alignment with *HsDIPP1* (PDB ID: 6WO7). The amino acids potentially important for Mg²⁺ and 5-PP-InsP₅ binding are labelled and shown as stick models. (D) Analogous to *HsDIPP1*, the *MoNudix* structure demonstrates a large positively charged site adjacent to the negatively charged Nudix hydrolase active site region. Surface electrostatic potential was calculated in ChimeraX⁶⁶ and is displayed in kcal/mol. (F) Purified recombinant protein (*MoNudix*, *MoNudix*^{E79Q}, *MoNudix*^{KKEE}, or *HsDIPP1*) at a concentration of 5 μ M or protein storage buffer was incubated with either inositol hexakisphosphate (InsP₆) or an inositol pyrophosphate (5-PP-InsP₅) (indicated along the top of the gel) for 60 minutes at 37 °C. The reaction products were separated using a polyacrylamide gel and visualised by staining with toluidine blue. (G) *MoNudix* with the protein surface coloured according to amino acid conservation across homologous effectors as determined by ConSurf.⁶⁵ The N-terminus of the crystal structure is truncated to remove three non-native amino acids resulting from the purification process. Next to the structure is the sequence of the *MoNudix* protein used to determine the crystal structure, with residue colouring indicating conservation. The amino acid residues potentially important for PP-InsP binding and hydrolysis are indicated with arrows. The two conserved, disulphide-bonded cysteines are indicated with yellow stars.

Magnaporthe and *Colletotrichum* Nudix effectors induce phosphate starvation responses in *N. benthamiana*

PP-InsPs play a crucial role in controlling the activation of PHRs by regulating their interaction with SPX proteins, thereby preventing PHRs from binding to DNA (Figure 3A).^{2,4,15,67} A reduction in intracellular PP-InsP concentration, as occurs when phosphate is limited, leads to the release and activation of PHRs, resulting in the transcription of phosphate starvation-induced (PSI) genes.³ If the *Magnaporthe* and *Colletotrichum* Nudix effectors hydrolyse PP-InsPs within plant cells, we predicted that the transcription of PSI genes will increase even under phosphate sufficient conditions (Figure 3A). To investigate if the Nudix effectors activate PHRs in plants, we expressed *MoNudix*, *ChNudix*, and the mutated proteins with compromised enzymatic activity (*MoNudix*^{E79Q} and *ChNudix*^{E78Q}) in *N. benthamiana* leaf tissue. We first analysed the mRNA levels of two genes (*NbSPX1* and *NbPEPC1*, primer sequences in Table S2) that are homologous to well-characterised *A. thaliana* PSI genes^{68,69} and contain multiple P1BS elements in their promoter region (Figure 3B, Table S3). The expression of *MoNudix* and *ChNudix* significantly increased the abundance of both *NbSPX1* and *NbPEPC1* mRNA, when compared to the expression of *MoNudix*^{E79Q} and *ChNudix*^{E78Q} (Figure 3C). Recently, Tang *et al.*, (2022) reported that *AtPHR1* activates the expression immunomodulatory RALF genes during phosphate starvation in *A. thaliana*. These RALF genes are characterised by a site-1 protease (S1P) cleavage site and a P1BS element within their promoter region.³⁵ S1P-processed RALF peptides negatively regulate plant immunity and suppress the immune-activated ROS burst.³⁷ We identified two potential *N. benthamiana* RALF genes homologous to the immune-suppressing *AtRALF23* and *AtRALF33*,³⁷ both with a S1P cleavage site and a P1BS element within their 3 kb promoter (Figure S5A, Table S3). Using qPCR, we demonstrate that the enzymatic activity of *ChNudix* significantly increases the mRNA abundance of both identified RALF genes (Figure S5B). The enzymatic activity of *MoNudix* significantly increases the mRNA abundance of one of the two RALF genes (Figure S5B), however, the increase for the second RALF gene was deemed insignificant by statistical testing (Figure S5B). Collectively, the qPCR results indicate that the hydrolase activity of *MoNudix* and *ChNudix* elevates the expression of PSI genes in *N. benthamiana* leaf tissue, consistent with the hydrolysis of PP-InsPs *in planta* by the *Magnaporthe* and *Colletotrichum* Nudix effectors.

To investigate this further, we developed a rapid *in planta* screening method to monitor PHR activation. In our system, the visible RUBY reporter gene is controlled by a promoter with multiple P1BS elements. RUBY converts tyrosine to red betalain, which can be visually

observed and quantified by extracting the betalain from plant tissue.⁷⁰ Therefore, in the assay, betalain production indicates activation of *N. benthamiana* PHRs. We utilised both a synthetic promoter sequence (PSI:RUBY) (Figure 3D, Table S3) and the promoter region from *NbPECP1* (PECP1:RUBY) (Table S3). When the PSI:RUBY or PECP1:RUBY gene alone is agroinfiltrated, no betalain production is observed (Figure 3E and 3F, Figure S5C and S5D). However, when co-transformed with the negative control genes, AvrM14-A, Nanoluciferase and the E-to-Q mutant Nudix proteins under the control of the 35S promoter, a low level of betalain production is detected (Figure 3E and 3F, Figure S5C and S5D). This suggests that overexpression of any gene using our system leads to a minor PHR activation in *N. benthamiana* leaf tissue, possibly due to excessive mRNA production depleting intracellular phosphate stores. Despite this, we demonstrate that co-transformation with six of the seven wild-type Nudix effectors significantly increases betalain production compared to the expression of the corresponding Nudix box (E-to-Q) mutant proteins and the negative controls (Figure 3E and 3F, Figure S5C and S5D). Additionally, we demonstrate that the *MoNudix*^{KKEE} mutation significantly (adjusted p-value ≤ 0.001) reduces betalain production compared to the wild-type protein (Figure 3E and F), which is consistent with the reduced InsP₆ binding and 5-PP-InsP₅ hydrolysis observed *in vitro*. Western blot analysis confirms the production of all tested effectors (Figure S1). Overall, our results demonstrate that the hydrolase activity of the *Magnaporthe* and *Colletotrichum* Nudix effector family increases the expression of genes controlled by *N. benthamiana* PHRs. These findings support the role of these effectors as PP-InsP hydrolases capable of inducing PSRs in plants.

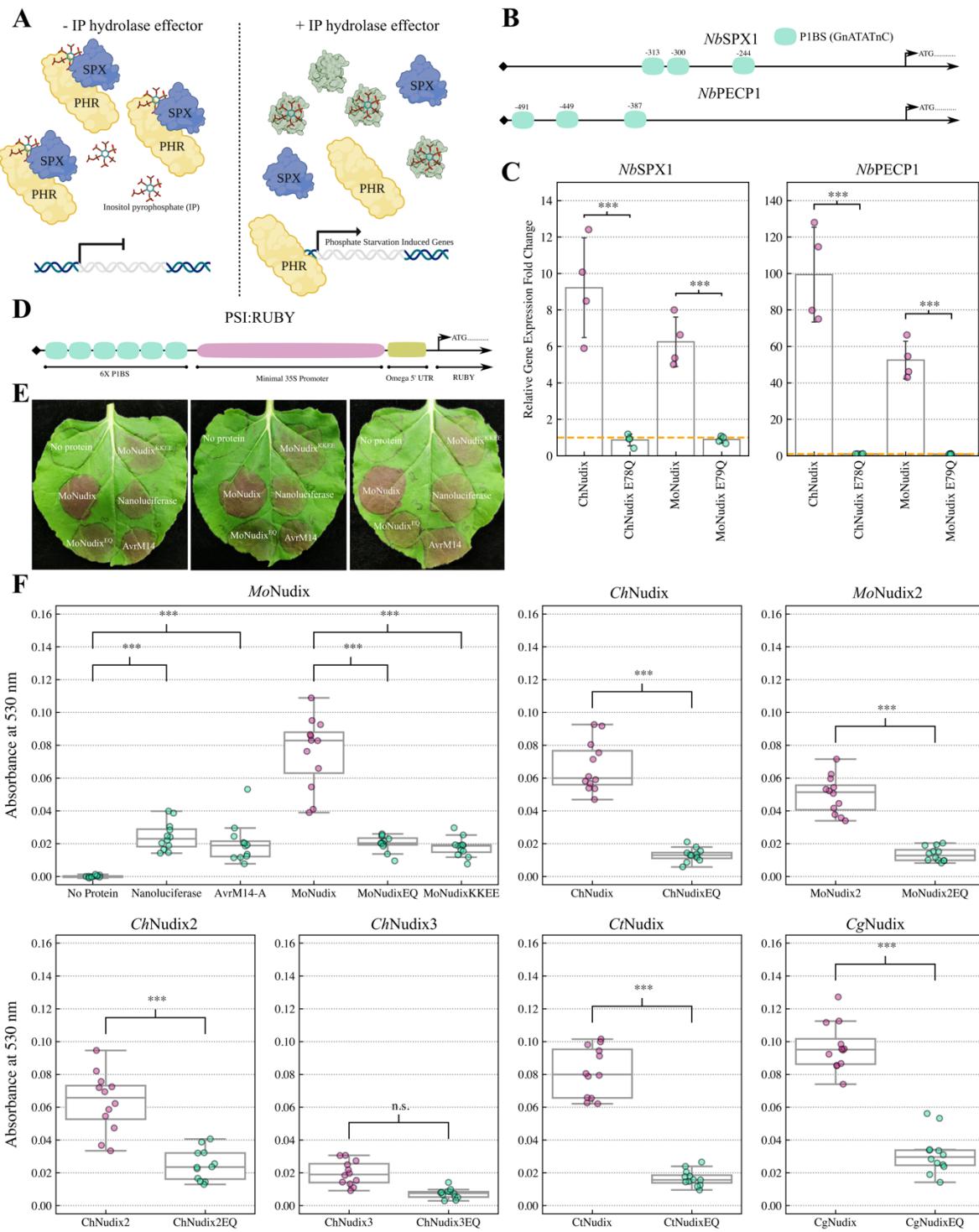


Figure 3: The *Magnaporthe* and *Colletotrichum* Nudix hydrolase effectors induce phosphate starvation responses in *Nicotiana benthamiana*. (A) A model for how a PP-InsP hydrolysing effector would activate the expression of phosphate starvation induced genes in plants by releasing PHRs from SPX proteins. (B) A schematic of the 500 base pairs upstream of the *NbSPX1* and *NbPECP1* start codons with the P1BS elements indicated. (C) RT-qPCR was completed on RNA extracted from *N. benthamiana* leaf tissue expressing either *MoNudix*, *ChNudix*, *MoNudix*^{E79Q}, *ChNudix*^{E78Q}, or leaf tissue transformed with an empty vector control. Values represent mean \pm SD across four biological replicates with individual data points shown as dots; *** p-value ≤ 0.001 (independent samples t-test). The relative gene expression fold change was calculated using the $2^{-\Delta\Delta Ct}$ method via comparison to the vector-only control treatment. The geometric mean of two reference genes was used for normalisation (*NbEF1a* and *NbUbe35*). The yellow dotted line indicates the relative expression level in the vector-only control treatment. (D) A schematic of the PSI:RUBY promoter and 5' UTR. The synthetic gene contains six P1BS elements in the promoter, a minimal 35 S sequence, and the Omega 5' UTR sequence from the Tobacco Mosaic Virus. Only the start of the RUBY CDS is depicted. Not to scale. (E) Representative leaf images demonstrating production of the red betalain pigment in leaf tissue co-transformed with the PSI:RUBY promoter/reporter and a Nudix effector or nanoluciferase (as labelled). (F) The absorbance at 530 nm of extracts from *N. benthamiana* leaf tissue co-transformed with the PSI:RUBY promoter/reporter with wild-type Nudix effectors and their corresponding mutant proteins (as labelled). There were 12 biological replicates for each treatment, as indicated by the dots overlaying the boxplots. In the boxplots, the horizontal line in the middle of the box represents the median value, and the box represents the interquartile range (IQR). To determine whether there was a significant difference between the treatments, a one-way ANOVA was completed followed by Tukey's post-hoc test; *** adjusted p-value ≤ 0.001 ; ** adjusted p-value ≤ 0.01 ; n.s. (not significant) adjusted p-value > 0.05 .

MoNudix promotes rice blast disease and inhibits rice immune responses

Both *MoNudix* and *ChNudix* use hydrolase activity to suppress the flg-22- and chitin-induced ROS burst in *N. benthamiana*. To further investigate the role of the PP-InsP hydrolase effectors in pathogen virulence and host immune suppression, we silenced *MoNudix* in *M. oryzae* using RNAi. We created two independent *M. oryzae* RNAi strains (RNAiNudix-1 and RNAiNudix-2), with a ~80% reduction in *MoNudix* gene expression in infected rice leaf sheaths at 28 hours post-inoculation (hpi) (Figure S6). Silencing of *MoNudix* resulted in a reduction of *M. oryzae* infection in whole plant spray inoculation assays (Figure 4A and 4B), indicating that the effector contributes to the virulence of the pathogen. Consistent with our experiments in *N. benthamiana*, silencing of *MoNudix* caused the accumulation of ROS in infected rice cells (Figure 4C and 4D), which coincided with restricted disease progression (Figure 4E).

Furthermore, confocal microscopy revealed that RNAiNudix-1 and RNAiNudix-2 strains triggered visible whole-cell and/or cell wall fluorescence in an average of 20 and 19%, respectively, of the host cells decorated by a single appressorium, as compared with only ~5% of those attacked by a single wild-type appressorium (Figures 4F and 4G). This result suggests that during infection the *MoNudix* effectors reduce the production of autofluorescent phenolic cell-wall components like lignin, that are induced by defense signalling. Collectively, our data demonstrate that *MoNudix* promotes the virulence of *M. oryzae* and the progression of rice blast disease, likely by suppressing rice defence responses.

Live-cell imaging studies have demonstrated that *M. oryzae* effectors are either delivered to the apoplast, the gaps between the fungal cell wall and host plasma membrane, or can be targeted to the host cell cytoplasm during infection.^{71,72} PP-InsPs are intracellular signalling molecules, therefore we hypothesise that *MoNudix* would function within the plant cell during infection. In the *M. oryzae*-rice pathosystem, cytoplasmic effectors are specifically localised within the biotrophic interfacial complex (BIC), which leads to the internalization of the effectors into the plant cell cytoplasm.⁷³⁻⁷⁶ Localization assays using *M. oryzae* strains expressing *MoNudix*:mRFP demonstrate that the Nudix hydrolase effector accumulates in BICs (Figure 4H), indicating that it functions as a cytoplasmic effector within the host plant cell. This localization pattern is consistent with sequence-based predictions⁷⁷ and with the predicted cytoplasmic localisation of plant Nudix hydrolases.^{78,79}

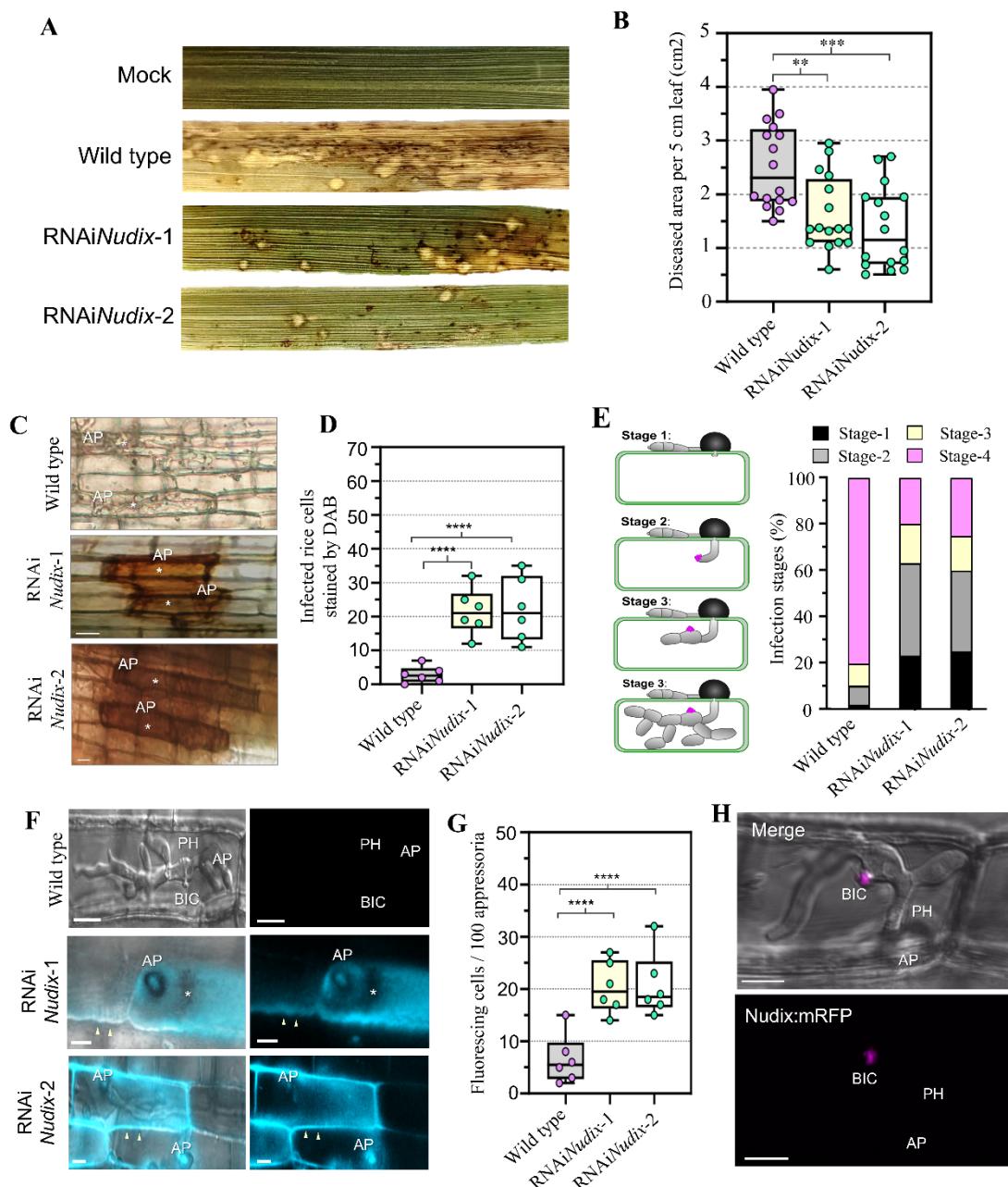


Figure 4: Silencing of *MoNudix* in *M. oryzae* indicates an important role in pathogen virulence and host immune suppression. (A) Whole plant spray inoculation assays with *M. oryzae* demonstrates that silencing *MoNudix* results in fewer, smaller lesions when compared to the wild-type control. (B) Quantification of the diseased area caused by wild type, RNAiNudix-1, and RNAiNudix-2 *M. oryzae* in 5 cm leaf segments of rice. Box and whisker plots with individual data points are shown; **P=0.0018, ***P=0.0002; n = 9 rice plants per replication. (C) DAB staining of penetrated plant cells at 32 hpi (hours-post inoculation). White asterisks

indicate infected rice cells. Scale bars = 10 μ m. (D) Percentages of infected cells stained by DAB. For each of the six replicates three sets of 100 cells were measured. ***P < 0.0001. (E) Quantification of four infection stages suggest a reduction of fungal virulence and colonization rate. For three replicates 100 infection sites were counted each. (F) Both wild-type and RNAi strains differentiated melanized appressoria (labelled AP) and invaded intact rice leaves, but the RNAi strains caused whole-cell (white asterisks) or cell wall fluorescence (white arrowheads) in rice under UV light, indicative of increased phenolic compound production and deposition in the cell wall. Scale bars = 10 μ m. (G) Quantification of fluorescing rice cells decorated with single appressoria of the wild-type or *Nudix*RNAi strains. For each of the six replicates three sets of 100 cells were measured. ***P < 0.0001. (H) *Nudix*:mRFP accumulates in BICs, which likely leads to its translocation into the rice cell cytoplasm. Images taken at 28 hpi. Image (top: merged bright field and mRFP fluorescence; bottom: mRFP fluorescence) shown as projections of confocal optical sections. Scale bars = 10 μ m.

Discussion

To monitor intracellular phosphate status, plant cells sense the level of PP-InsPs. In response to a lack of PP-InsPs, plants initiate a PSR that is predominantly mediated by the PHR family of transcription factors. In this study, we have identified a conserved family of PSR-inducing PP-InsP hydrolase effectors in *Magnaporthe* and *Colletotrichum* pathogenic fungi. Additionally, we demonstrate that the silencing of one PP-InsP hydrolase effector in *M. oryzae* significantly impairs the progression of rice blast disease.

Phosphate starvation and plant-microbe interactions

Plant phosphate status plays a critical role in establishing symbiosis with mycorrhizal fungi,^{32,33} and in regulating interactions between *A. thaliana* and the root endophyte *Colletotrichum tofieldiae*.²⁹⁻³¹ Phosphate starvation negatively regulates some plant immune responses, possibly to facilitate symbiosis with beneficial soil microbes,^{34,35} and/or to prevent immune-related suppression of Pi transport.³⁶ Our findings indicate that pathogenic *Magnaporthe* and *Colletotrichum* species employ PP-InsP hydrolase effectors which manipulate plant phosphate sensing mechanisms and inhibit immune responses. We demonstrate that the PP-InsP hydrolase effectors inhibit defence-related ROS production both during the infection process and when expressed in *N. benthamiana*. Suppression of ROS may occur due to the activation of PHRs. In *A. thaliana*, *AtPHR1* negatively regulates immune responses triggered by flg-22,³⁴ potentially via the increased production of RALF peptides.³⁵ Immunomodulatory RALFs are perceived by the receptor kinase FERONIA, which acts as a scaffolding protein to regulate immune receptor complex formation.³⁷ In support of PHR-activated RALF/FERONIA-mediated ROS suppression, we observe an increase in the expression of two *N. benthamiana* RALF genes with a P1BS element in their 3kb promoter region, facilitated by the enzymatic activity of the effectors. Tang *et al.*, (2022) reported 17 RALF genes in rice with at least one P1BS element in their 2 kb promoter region.³⁵ Further investigation is required to determine whether these rice RALFs act as negative-regulators of plant immunity, if their expression is controlled by PHRs, and if the PP-InsP hydrolase effectors from *M. oryzae* promote their production to inhibit immune signalling and enhance infection. The suppression of innate immune responses such as ROS production, may explain the significant contribution *MoNudix* makes to the severity of rice blast disease observed in our infection assays. Additionally, the increase in bioavailable intracellular phosphate that occurs during a PSR could enhance pathogen growth. Supporting this hypothesis, transcriptomic analysis suggests that *C.*

fructicola is starved of phosphate during initial biotrophic growth,⁸⁰ and infection assays indicate that excessive phosphate supply promotes *M. oryzae* virulence on rice.⁴²

Other roles of PP-InsPs in plants

While PP-InsPs play a critical role in regulating PHRs, they also possess other important signalling roles in plants cells, which could contribute to the virulence function of PP-InsP hydrolase effectors. PP-InsPs are involved in signalling by the phytohormones auxin and jasmonate. The jasmonate receptor complex consisting of CORONATINE INSENSITIVE 1 (COI1) and a JASMONATE ZIM DOMAIN (JAZ) protein, requires a PP-InsP co-factor to detect the bioactive form of jasmonate, JA-Ile ((3R,7S)-jasmonoyl-L-isoleucine).^{81,82} Similarly, the auxin receptor complex consisting of TRANSPORT INHIBITOR RESPONSE1 (TIR1), ARABIDOPSIS SKP1 HOMOLOG 1 (ASK1), and an AUXIN RESISTANT/INDOLE-3-ACETIC ACID-INDUCIBLE (Aux/IAA) transcriptional repressor also requires a PP-InsP co-factor.^{83,84} By manipulating intracellular PP-InsP levels, the *Magnaporthe* and *Colletotrichum* effectors may influence jasmonate and auxin signalling pathways, both of which have been identified as likely targets of previously studied effectors.⁸⁵⁻⁹⁰

While our results indicate that these effectors hydrolyse PP-InsPs both *in vitro* and *in planta*, further research is required to isolate which of the various functions of PP-InsPs are important for the virulence of pathogenic *Magnaporthe* and *Colletotrichum* fungi. Identifying if the effectors preferentially hydrolyse a specific PP-InsP isomer/s *in planta* may provide further insight into their virulence function.

Which PP-InsP isomer is hydrolysed by the *Magnaporthe* and *Colletotrichum* effectors *in planta*?

The presence of at least four distinct PP-InsP compounds in plants, 1/3-PP-InsP₅, 5-PP-InsP₅, 4/6-PP-InsP₅, and 1/3,5-PP-InsP₄ (commonly referred to as InsP₈), is supported by experimental evidence.⁶ However, in our study, we exclusively examined the ability of the *Magnaporthe* and *Colletotrichum* effectors to hydrolyse 5-PP-InsP₅. This limitation is primarily due to the commercial unavailability of PP-InsPs. Nevertheless, our *in planta* findings demonstrate the ability of these effectors to activate PHRs, strongly suggesting that at least 5-PP-InsP₅ and/or InsP₈ are hydrolysed by the effectors. InsP₈ mediates SPX binding to PHRs,^{3,4} and its levels are likely dependent on the availability of 5-PP-InsP₅.⁶ Consistently, plants unable to synthesise 5-PP-InsP₅ and InsP₈ demonstrate a PHR-dependent constitutive

PSR.^{3,5,6,91} Therefore, the hydrolysis of either compound by the effectors would explain the observed PHR activation. The structure of *MoNudix* indicates that the binding site can accommodate either 5-PP-InsP₅ or InsP₈. Intriguingly, we predict that the additional phosphate moiety in InsP₈ would interact with the highly conserved arginine-35 residue and may enhance binding affinity, suggesting a potential preference for InsP₈ over 5-PP-InsP₅. To precisely identify the targeted PP-InsP/s, future studies could use the recently developed capillary electrophoresis coupled to electrospray ionization mass spectrometry (CE-ESI-MS) method to analyse PP-InsP extracts from plant tissue expressing the effectors.⁹²

A model for the biological function of the PP-InsP hydrolase effector family

Our live-cell imaging results demonstrate that RFP-tagged *MoNudix* localises to the BIC during infection. BIC localisation suggests that the *MoNudix* effector is translocated into the host cytoplasm and does not function as an apoplastic effector.^{72,73,93} This contrasts with a previous study on the sequence-related *CtNudix*,⁴⁴ which suggested that the effector would function in the apoplast, as expression of the effector in *N. tabacum* with a signal peptide results in cell death. The decision of Bhadauria *et al.*, (2013) to produce the effector with a signal peptide was not informed by protein tracking during infection and therefore the cell death phenotype when the effector is secreted in *N. tabacum* is potentially unrelated to its virulence function during infection.

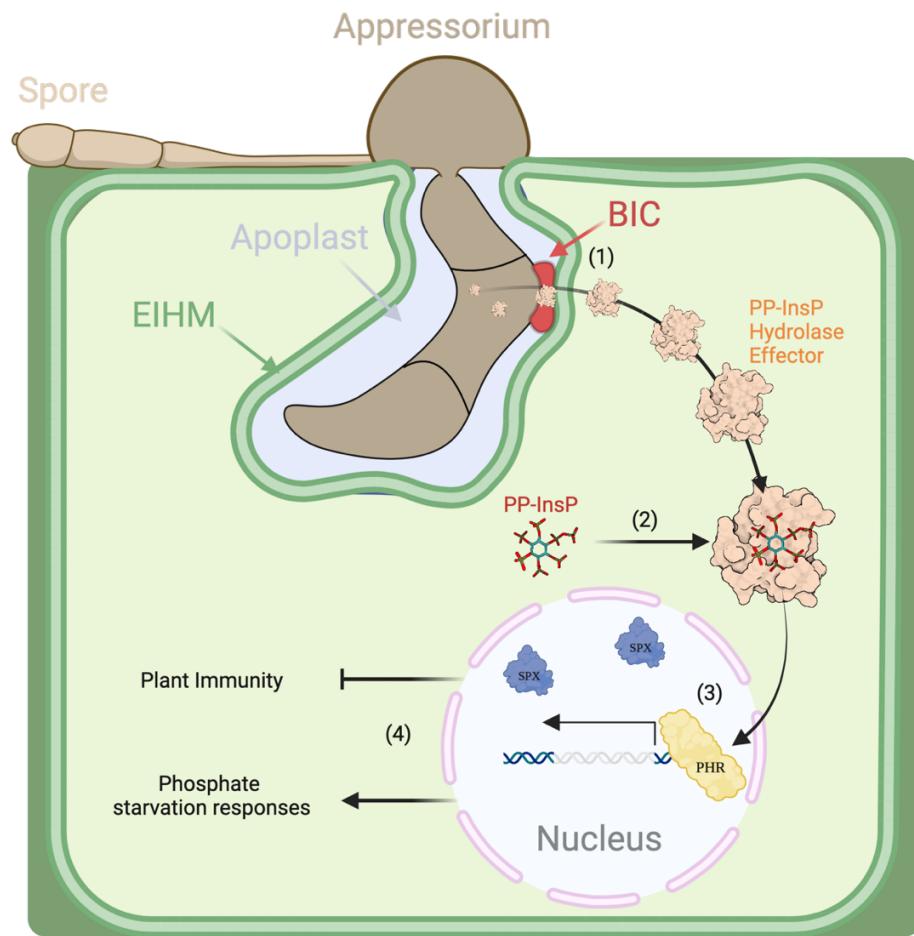


Figure 5: A model for the virulence function of the *Magnaporthe* and *Colletotrichum* Nudix hydrolase effectors. (1) The effectors are secreted from the invading fungus into the host plant cell cytoplasm. (2) The effectors act as enzymes, hydrolysing the pyrophosphate bonds on inositol pyrophosphate signalling molecules. (3) The loss of inositol pyrophosphates prevents SPX-mediated inhibition of PHRs, resulting in the transcription of phosphate starvation inducible genes. (4) Phosphate starvation responses are activated, including those that suppress plant immune responses.

On the basis of our data, we propose a model by which the *Magnaporthe* and *Colletotrichum* Nudix effectors, including *CtNudix*, are translocated into their respective host plant cells (Figure 5). Once inside, the effectors function as PP-InsP hydrolase enzymes, reducing the availability of plant PP-InsPs and subsequently activating PHRs (Figure 5). This induces a plethora of transcriptional changes, some resulting in the suppression of immune responses like ROS production and lignin deposition (Figure 5). Ultimately, the immune suppression promotes disease progression.

Acknowledgments

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Figures 3A and 5 were created with BioRender.com.

Author contributions

C.L.M, J.R.G, E.O-G, and S.J.W designed the experiments. C.L.M, C.S.Z., S.A, S.d.P, E.E, and S.S performed the experiments. C.L.M, C.S.Z, S.A, J.R.G, D.J.E, E.O-G, and S.J.W analysed the data. C.L.M wrote the manuscript with input from all authors.

Declaration of interests

The authors declare no competing interests.

Methods

Resource availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Simon J. Williams (simon.williams@anu.edu.au).

Materials availability

All requests for the plasmids and purified proteins should be directed to and will be fulfilled by the lead contact, Simon J. Williams (simon.williams@anu.edu.au).

Data and code availability

- Atomic coordinates and structure factor amplitudes for *MoNudix* have been deposited in the Protein Data Bank (PDB) under accession code 8SXS. The diffraction images have been uploaded to proteindiffraction.org.
- This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

Experimental model and subject details

Nicotiana benthamiana

Nicotiana benthamiana plants were grown in a growth room at 25 °C under a 16-hour/8-hour light/dark photoperiod. Fully expanded leaves from 5-week-old plants were used for *Agrobacterium*-mediated transient gene expression for qPCR and RUBY promoter/reporter assays.

Oryza sativa

Rice (*O. sativa*) plants for both whole plant spray inoculation and detached leaf sheath assays were grown in Bacto Top Soil (Michigan Peat Co., Houston, Texas) in a Caron 7301-50 Plant Growth Chamber with equal numbers of fluorescent lamps (Philips ED37, 400 W). At rice seedling height, ~1 m from the bulbs, light ranged in intensity from 600 to 1,200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were grown at ~70% relative humidity under a daily cycle of 12 h of light at 28°C and 12 h of darkness at 24°C. At 2 weeks, plants are fertilized with Jack's Professional Peat Lite 20-10-20 Fertilizer (#77860; JR Peters, Inc.; Allentown, PA). Whole plant infection assays were performed by spray inoculation of 2–3-week-old rice plants as previously described.⁷²

Fungal strains

Fungal strains (*M. oryzae*) were stored on dried filter papers at -20°C and cultured on rice bran agar plates⁹⁴ at 25°C for up to 2 weeks under continuous light in Percival Scientific (Model

CU-36L5) tissue culture incubators equipped with one half fluorescent lights (FT20T12/cw, 20W) and one-half black lights (FT20T12/BL, 20W).

Bacterial strains

E. coli NEB® 5-alpha and *Agrobacterium tumefaciens* strain GV3101 were grown in LB media with 220 rpm shaking at 37 °C and 28 °C, respectively. Appropriate antibiotic concentrations when used for *E. coli* and *Agrobacterium tumefaciens* growth were ampicillin 100 µg/mL, kanamycin 50 µg/mL, spectinomycin 50 µg/mL, gentamycin 25 µg/mL and rifampicin 50 µg/mL.

For protein expression in Shuffle® T7 express *E. coli* the growth conditions were terrific broth (TB) media at 30 °C with 50 µg/mL kanamycin. For protein expression in *E. coli* BL21 (DE3) the growth conditions were ZYM-5052 media⁹⁵ at 37 °C with 50 µg/mL kanamycin.

Method details

Plasmid construction

All effector gene sequences had their predicted signal peptide⁹⁶ removed, were codon optimised for *E. coli* and ordered from Integrated DNA technologies, Inc IDT® as double-stranded DNA fragments. The DNA fragments were cloned into either a modified pOPIN plasmid⁹⁷ with a T7 promoter and a 6xHIS 3C protease site N-terminal tag⁹⁷ if used for protein expression in *E. coli*, or a level 1 MoClo plasmid⁹⁸ along with a 35 S promoter, Omega 5' UTR translational enhancer, 3xHA N-terminal tag, and octopine synthase terminator if used for expression in *N. benthamiana*. All level 1 MoClo genes were subsequently inserted into the level 2 acceptor plasmid pICSL4723 before transformation into *Agrobacterium tumefaciens* (GV3101).

The RUBY polyprotein gene sequence⁹⁹, and all gene promoter sequences used in this study (listed in Table S3) were ordered from Integrated DNA technologies, Inc IDT® as double-stranded DNA fragments and cloned into level 0 MoClo plasmids⁹⁸, before being used to create level 1 and level 2 plasmids. *Entamoeba histolytica* IP6KA and *Homo sapiens* DIPP1 were ordered as *E. coli*-codon optimised double-stranded DNA fragments and cloned into the modified pOPIN⁹⁷ plasmid with a T7 promoter and a 6xHIS 3C protease site N-terminal tag. All gene sequences had any BbsI and BsaI cleavage sites removed without altering protein sequence to enable GoldenGate assembly.

Protein expression and purification

AvrM14-A, *At*Nudx7, and *At*Nudx7^{E154Q} proteins were purified previously.⁴⁶ *Eh*IP6KA and *Hs*DIPP1 were expressed in *E. coli* BL21 (DE3) cells using ZYM-5052 autoinduction media.⁹⁵

Cells were grown by continuous shaking at 37 °C until the OD_{600nm} reached 0.6 – 0.8. The temperature was then dropped to 18 °C and cells were incubated with shaking for another 18 hours before harvesting via centrifugation.

MoNudix, *MoNudix2*, and *ChNudix* proteins (wild-type and mutants) were expressed in *E. coli* Shuffle® cells grown in Terrific Broth. Cells were grown by continuous shaking at 30 °C until the OD_{600nm} reached 0.6 – 0.8. The temperature was then dropped to 16 °C, IPTG was added to a final concentration of 200 μM, and incubation with shaking continued for another 18 hours before harvesting via centrifugation.

Following centrifugation, all cell pellets were resuspended in lysis buffer (50 mM HEPES pH 8.0 (*MoNudix*, *MoNudix*^{E79Q}, *MoNudix*^{KKKE}), pH 7.5 (*MoNudix2*, *MoNudix2*^{EQ} *ChNudix*, *ChNudix*^{E78Q}), or pH 7.0 (*HsDIPP1*, *EhIP6KA*), 150 mM NaCl, 1 mM PMSF, 1 μg ml⁻¹ DNase, and 1 mM DTT (DTT only included for *EhIP6KA* and *HsDIPP1*)). All cells were lysed using sonication and cellular debris pelleted by centrifugation. The resulting supernatant was applied to a 5 mL HisTrap FF crude column (Cytiva, Marlborough, Massachusetts). To remove loosely bound proteins, the column was washed with the lysis buffer without PMSF or DNase containing 30 mM imidazole. The remaining bound proteins were eluted with a continuous gradient of imidazole from 30 mM to 250 mM over 10 minutes, using an Äkta pure chromatography system. Fractions were analysed by Coomassie-stained SDS-PAGE and fractions containing the protein of interest were pooled, dialysed to remove the imidazole, and incubated with recombinant 6xHis-tagged 3C protease overnight at 4 °C (except *EhIP6KA*, which was stored following dialysis without 3C protease incubation). The protein of interest was separated from any uncleaved protein, the fusion tag, and 3C protease by immobilized metal affinity chromatography and purified further by size-exclusion chromatography (SEC) using either a HiLoad 16/600 Superdex® 75 pg or a HiLoad 26/600 Superdex® 75 pg column pre-equilibrated in buffer (10 mM HEPES (pH same as in corresponding lysis buffer), and 150 mM NaCl). After SEC, fractions containing the protein of interest were identified using SDS-PAGE and concentrated using Amicon® Ultra Centrifugal filters (Merck, Darmstadt, Germany) before storage at -80 °C. The sequences of all purified proteins used in this study are listed in Table S4.

Putative Nudix hydrolase effector identification

To identify putative Nudix hydrolase effectors homologous to *MoNudix*, the NCBI protein database¹⁰⁰ was searched using blastp with the protein sequence of *MoNudix* (default parameters; word size = 5, expect threshold = 0.05). Any identified hits without a predicted signal peptide or Nudix hydrolase domain were filtered out by screening the sequences using

SignalP6.0⁹⁶ and InterProScan.¹⁰¹ To reduce the length of the list while retaining sequence diversity, if two or more sequences shared > 95% sequence identity, only one sequence was selected at random to remain in the list presented in Table S1.

PhyML⁶³ (version 3.3) was used to estimate a maximum-likelihood phylogeny with selected protein sequences from this list, the resulting phylogeny was visualised using iTOL⁶⁴ (version 6.7.6).

Agroinfiltrations

Agrobacterium tumefaciens (GV3101) with the desired plasmid was suspended in infiltration buffer (10 mM MES pH 5.6, 10 mM MgCl₂ and 200 μM acetosyringone) to an optical density at 600 nm (OD_{600nm}) of 0.5 (for PSI:RUBY co-infiltrations) or 1.0 (for qPCR and the ROS burst assay). For co-infiltrations, *Agrobacterium tumefaciens* (GV3101) with a PSI:RUBY promoter/reporter plasmid was added to the infiltration buffer to an OD_{600nm} of 0.5. The final combined OD_{600nm} was 1.0 for all infiltrations. All cultures were incubated in the dark at 28 °C with 220 rpm shaking for 2 to 3 hours before syringe-infiltration into *N. benthamiana* leaves. Infiltrated plants were kept in the same growing conditions as before infiltration.

ROS burst assays

Measurement of ROS was completed as described previously with some minor modifications.¹⁰² In brief, *N. benthamiana* leaf discs (4 mm diameter) were floated on water overnight in a 96-well plate. The water was replaced with an elicitor solution (200 μM luminol, 20 μg ml⁻¹ horseradish peroxidase and 100 nM flg-22 or 5 μg ml⁻¹ chitin), and luminescence was measured over time using a Tecan Infinite® M Plex (Tecan, Männedorf, Switzerland) plate reader at room temperature.

Immunoblot analysis

N. benthamiana leaf tissue was frozen in liquid nitrogen and ground into a fine powder. Soluble protein was extracted by adding an equal volume of lysis buffer (50 mM HEPES (pH 7.5), 1 mM EDTA, 2% PVPP, 5 mM DTT, 1 mM PMSF). The samples were mixed by rotating at 4 °C for 2 minutes, prior to centrifugation at 4 °C for 15 minutes at 17 000 xg and the supernatant was collected. Approximately 20 μg of each protein solution was separated on two 15% SDS-PAGE gels. The first gel was stained with Coomassie blue to assess protein loading across samples, while proteins from the second gel were transferred onto a 0.22 μM nitrocellulose membrane. Blots were probed with HRP-conjugated mouse anti HA-tag (1:2000 dilution) either from ABclonal (Woburn, Massachusetts) or Roche (Basel, Switzerland). Pierce™ ECL substrate (Thermo Fisher Scientific, Waltham, Massachusetts) was added to the immunoblots and chemiluminescence detected using a ChemiDoc imager (Bio-Rad, Hercules, California).

General substrate screening and mRNA decapping assays

The phosphomolybdate Nudix hydrolase enzyme assays and the mRNA decapping assays were performed as described previously.⁴⁶

Inositol pyrophosphate hydrolysis assays

To assess inositol pyrophosphohydrolase activity 5-PP-InsP₅ was first synthesised in a 500 µL reaction volume following previously described methods¹⁰³ using the purified *EhIP6KA* protein. The resulting 5-PP-InsP₅ was purified using the previously described gel electrophoresis-based method.¹⁰⁴ Purified 5-PP-InsP₅ was incubated with 5 µM recombinant protein in 50 mM Tri-HCl pH 8.0, 5 mM MgCl₂ at 37 °C for 60 minutes. After incubation the reaction products were separated and visualised using previously described methods¹⁰⁵ with minor modifications (we utilised a smaller gel (8.3 x 7.3 x 0.1 cm) and ran the gel at 300V for approximately 5 hours, until the dye front was 2/3 of the way through the gel).

Protein crystallisation and structure determination

Crystallisation screening with purified *MoNudix* protein (residues 35 to 156) was conducted using a Mosquito robot (STP LabTech, Melbourn, UK) in a 96-well plate format using sparse matrix screens. The sitting drop vapour-diffusion method of crystallization was used and drops consisting of 100 nL 30 mg/mL *MoNudix* containing 18 mM InsP₆ (Merck, Darmstadt, Germany) combined with 100 nL reservoir solution were equilibrated against 100 µL reservoir solution. The reservoir solution resulting in the *MoNudix* crystals analysed in the study was 200 mM Potassium thiocyanate with 20% PEG3350 (from the SG1™ Screen (Molecular Dimensions, Newmarket, United Kingdom)). To create the cryoprotectant 80 µL of the reservoir solution was combined with 10 µL of glycerol and 10 µL of ethylene glycol. The crystal was transferred to the cryoprotectant before flash cooling in liquid nitrogen. Diffraction datasets were collected on the MX2 beamline at the Australian Synchrotron.¹⁰⁶ The highest resolution dataset allowed by the beamline geometry was selected for processing in XDS, and then scaled using AIMLESS in the CCP4 suite.^{107,108} The *MoNudix* crystal structure was determined using maximum-likelihood molecular replacement (MR) with Phaser in Phenix.^{109,110} The MR search model was an AlphaFold⁵⁹ model of the *MoNudix* sequence used for crystallisation (sequence in Table S4). For all datasets automated model building and initial refinement was completed using Phenix AutoBuild.¹¹¹ Subsequent model building was carried out manually in Coot¹¹² in-between rounds of automated refinement using Phenix Refine¹¹³. Analysis of the final structures was performed with Coot¹¹², ChimeraX⁶⁶, and ConSurf⁶⁵ (default parameters were used for analysis). Map coordinates and structure files have been deposited in the Protein Data Bank under ID 8SXS, summary statistics are listed in Table S5.

Micro-scale Thermophoresis

MST experiments were performed on a Monolith.NT115 instrument (NanoTemper Technologies, Munich, Germany) at 25 °C. MoNudix and MoNudix^{KKEE} were labelled with Alexa Flour 647 succinimidyl ester (Thermo Fisher Scientific) and used at a final concentration of 50 nM. In brief, 20 µM of the protein solution was incubated with 2-fold molar excess of the fluorophore for 2 hours in the dark at room temperature, and the free dye was subsequently removed using a PD-10 desalting column (Cytiva). A stock solution of InsP₆ was serially diluted in buffer (10 mM HEPES pH 8.0, 150 mM NaCl), mixed 1:1 with the labelled protein and loaded into standard capillaries (NanoTemper Technologies). MST measurements were recorded using 20 % LED power and 20 to 80 % MST power and analysed using MO. Affinity Analysis software 2.2.7 (NanoTemper Technologies).

RNA Extractions and RT-qPCR

At 3 days post-infiltration (dpi) approx. 100 mg of *N. benthamiana* leaf tissue was collected from each infiltration site and frozen in liquid nitrogen. For RNA extraction and purification, the Monarch® Total RNA Miniprep Kit (NEB, Ipswich, Massachusetts) was used following the recommended protocol for plant tissue, with tissue lysis achieved by grinding the plant tissue into a fine powder while frozen in liquid nitrogen.

cDNA synthesis from the purified RNA was achieved using the LunaScript® RT SuperMix Kit (NEB). qRT-PCR was performed using the Luna® Universal qPCR Master Mix (NEB) on a ViiA 7 PCR System (Applied Biosystems, Waltham, Massachusetts), with primers listed in Table S2. Relative expression levels were calculated using the 2^{-ΔΔCt} method,¹¹⁴ accounting for the PCR efficiency of each primer,¹¹⁵ and normalised to the geometric mean¹¹⁶ of the internal controls, *NbUbe35*, and *NbEIF1a*.

Betalain extraction and measurement

We used a modified version of a previous betalain extraction method.⁹⁹ In brief, at 3 dpi six 4 mm leaf disks were excised from the infiltrated area, incubated for 30 minutes with 30 rpm rotation in 1 mL 50% methanol, and then 200 µL from each solution was transferred into a transparent 96-well plate and absorbance at 530 nm was measured. All values were zeroed using the absorbance measurement of 50% methanol alone.

Live-cell imaging of Nudix:mRFP *M. oryzae* and the RNAi strains *in planta*

Rice leaf sheath inoculations were performed as described¹¹⁷ with the following modification. We used sheath pieces that were thinner trimmed sheaths (~3 cell layers thick). Susceptible rice variety YT-16 was used unless otherwise mentioned. Briefly, 7-cm long leaf sheath pieces from 3-week-old plants were placed in a sealable Pyrex glass moist chamber. Leaf sheath

sections were placed on inverted 8-well PCR tube strips to avoid contact with wet paper and to hold the epidermal cells directly above the mid-vein horizontally flat for the uniform distribution of inoculum in trimmed leaf sheath pieces.⁷⁶ A spore suspension (104 spores/ml in sterile 0.25% gelatin, Cat. #G-6650, Sigma-Aldrich) was prepared from 10-day-old cultures and injected into one end of the sheath using a 100-ml pipette. Each segment was trimmed at 18 to 30 hours post inoculation (hpi), and imaged immediately by laser confocal microscopy. Biological replicates were independent experiments performed with fungal cultures fresh out of frozen storage and with new rice plants. All conclusions are supported by at least 3 biological reps, with each replication including observation of ~100 infection sites. Confocal imaging was performed with a Leica SP8 confocal microscope system using two water immersion objectives, C-Apochromat 40x/1.2 WCorr. and C-Apochromat 63x/1.2 WCorr. Excitation/emission wavelengths were 543 nm/560–615 nm for mRFP, and were 358 nm/461 nm for cell wall fluorescence. Image acquisition and processing were done using Leica LAS X 2020 software. For ROS analysis, rice leaves inoculated with conidium suspensions (3x10⁵ spores/mL) of the wild-type and RNAi strains were stained with DAB at 32 hpi (hours-post inoculation).

ROS detection *in planta*

Host-derived ROS was observed by staining with DAB (Sigma-Aldrich) as described previously.¹¹⁸ Rice leaves inoculated with RNAi and wild type strains at 30 hpi were incubated in 1mg/mL DAB solution, pH 3.8, at room temperature for 8 hours and destained with ethanol:acetic acid solution (94:4, v/v) for 1 hour.¹¹⁹

Generation of RNAi strains in *M. oryzae*.

The RNAi cassette from plasmid pRedi¹²⁰ was used to generate an RNAi construct targeting *MoNudix* transcripts. The 312-bp sense and antisense fragments were amplified from genomic DNA of *M. oryzae*, using the primers RNAi(Nudix1)-fw and RNAi(Nudix1)-Rv, and RNAi(Nudix1)i-fw and RNAi(Nudix1)i-Rv, respectively (sequences listed in Table S2). The sense and antisense fragments were used to replace the XhoI-SnaBI and BglII-ApaI fragments of pRedi and were thus separated by 135 bp of the intron of the *M. oryzae* Cut2 gene (National Center for Biotechnology Information: XM_365241.1), existing in pRedi, as a linker.¹²⁰ Plasmid inserts were subsequently sequenced to verify sequence accuracy. The resulting 6.0-kb RNAi construct was excised from pRedi by DraI digestion, purified by gel elution, and transformed into conidial protoplasts of *M. oryzae*, and single spore isolates were generated.¹¹⁷ Knockdown of the target genes was confirmed by qRT-PCR¹²¹ at 28 hours post inoculation. At

this point, rice plants showing satisfactory reduction of the transcription levels were used in our standard conidial spray inoculation and leaf sheath assays.

Statistics for *M. oryzae* experiments

All experiments were performed with at least 3 biological replications, which are independent experiments with fungal cultures directly growing out from frozen storage and different rice plantings. Biological replications included at least two technical repeats (independent assays with the same biological materials) for further confirming reproducibility of the data. The sample sizes, number of biological replicates, and the statistical tests used in each experiment are specified in the figure legends. Data were analysed using an unpaired two-tailed Student's t-test. $P < 0.05$ was considered significant and exact values are shown where appropriate. All statistical analysis was performed using R Statistical Software (version 4.1.2) and Prism9 (GraphPad). Dot plots were routinely used to show individual data points and generated using Prism9 (GraphPad). Bar graphs show the mean \pm s.e.m. (unless stated otherwise) and were generated using Prism9 (GraphPad). Analysis of datasets are represented by box-and-whisker plots that show the 25th and 75th percentiles, the median indicated by a horizontal line, and the minimum and maximum values indicated by the ends of the whiskers.

Supplemental information

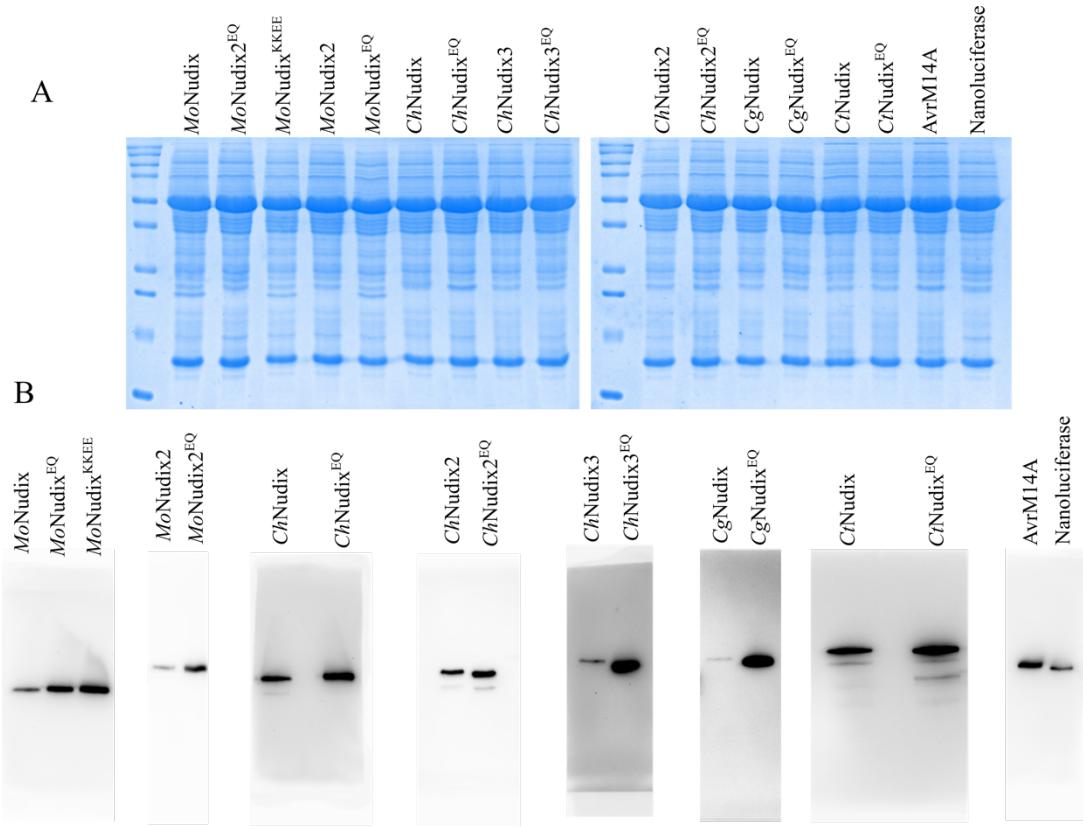


Figure S1: Western blots of soluble protein extracts demonstrates production of all effector proteins in *N. benthamiana* leaves. Related to Figure 1 and Figure 3. (A) Coomassie-stained SDS-PAGE protein gels demonstrate equivalent total protein amounts in the soluble *N. benthamiana* protein extracts from the agroinfiltrated plant tissue used for western blotting. The effector that should be present in each sample is indicated along the top of the gel; the first lane of both gels contains the Precision Plus Protein Dual Color Standards (Biorad, Hercules, California) (B) Total protein extracts from *N. benthamiana* leaf tissue agroinfiltrated with a construct to express a HA-tagged effector, as labelled along the top of each blot, were analysed by western blotting. Blots were probed with mouse anti-HA HRP-conjugated antibodies.

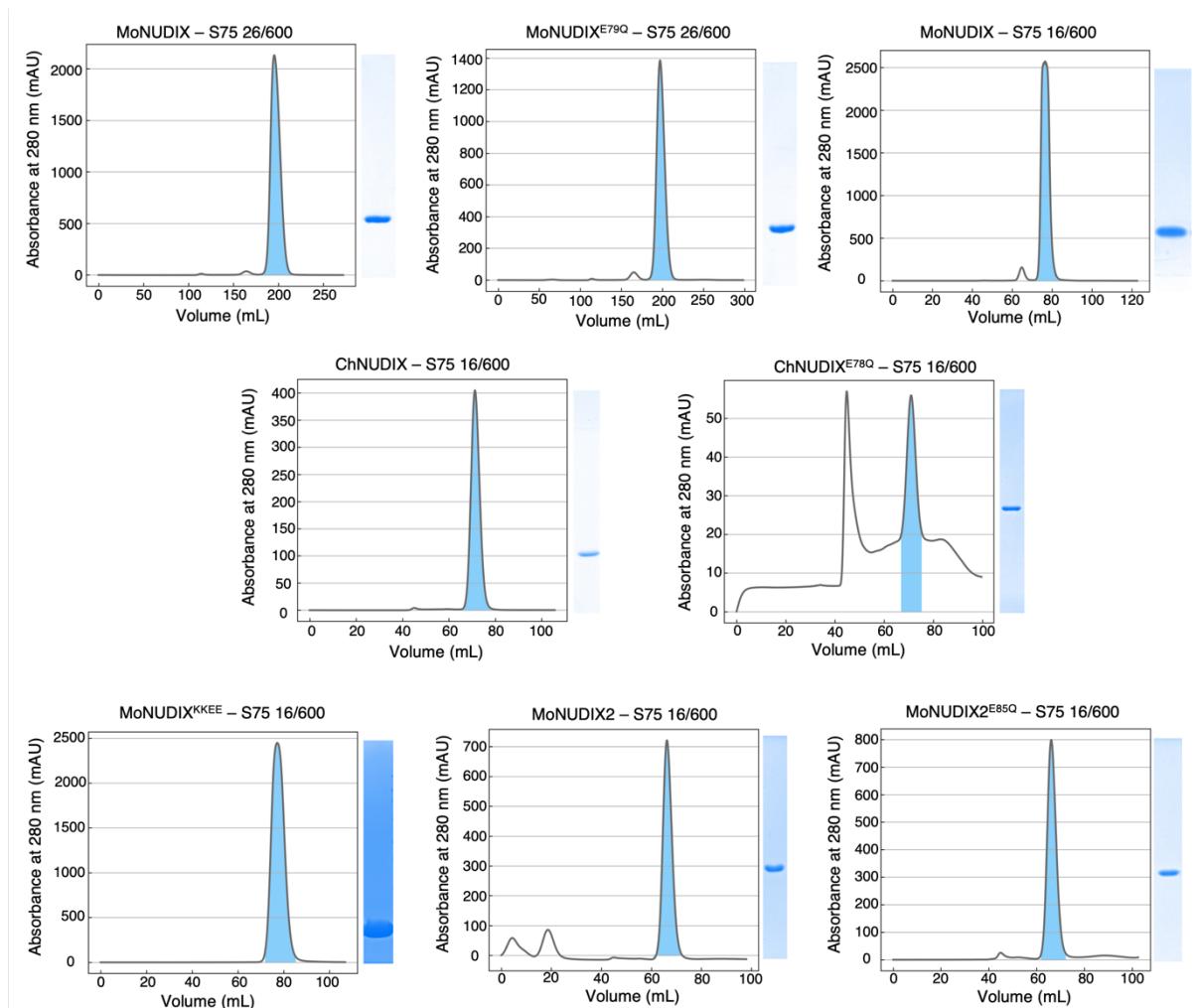


Figure S2: All effector proteins used in enzymatic assays were purified to homogeneity. Related to Figure 1, Figure 2, and Figure S3. Size exclusion chromatography (SEC) profiles for all effector proteins purified in this study. The label at the top of each profile indicates the protein and the Highload Superdex column (Cytiva) used in the purification process. The area under the peak shaded blue indicates the volume collected for each effector. Alongside each profile is a Coomassie-stained SDS-PAGE gel demonstrating the purity of the final protein sample collected following SEC.

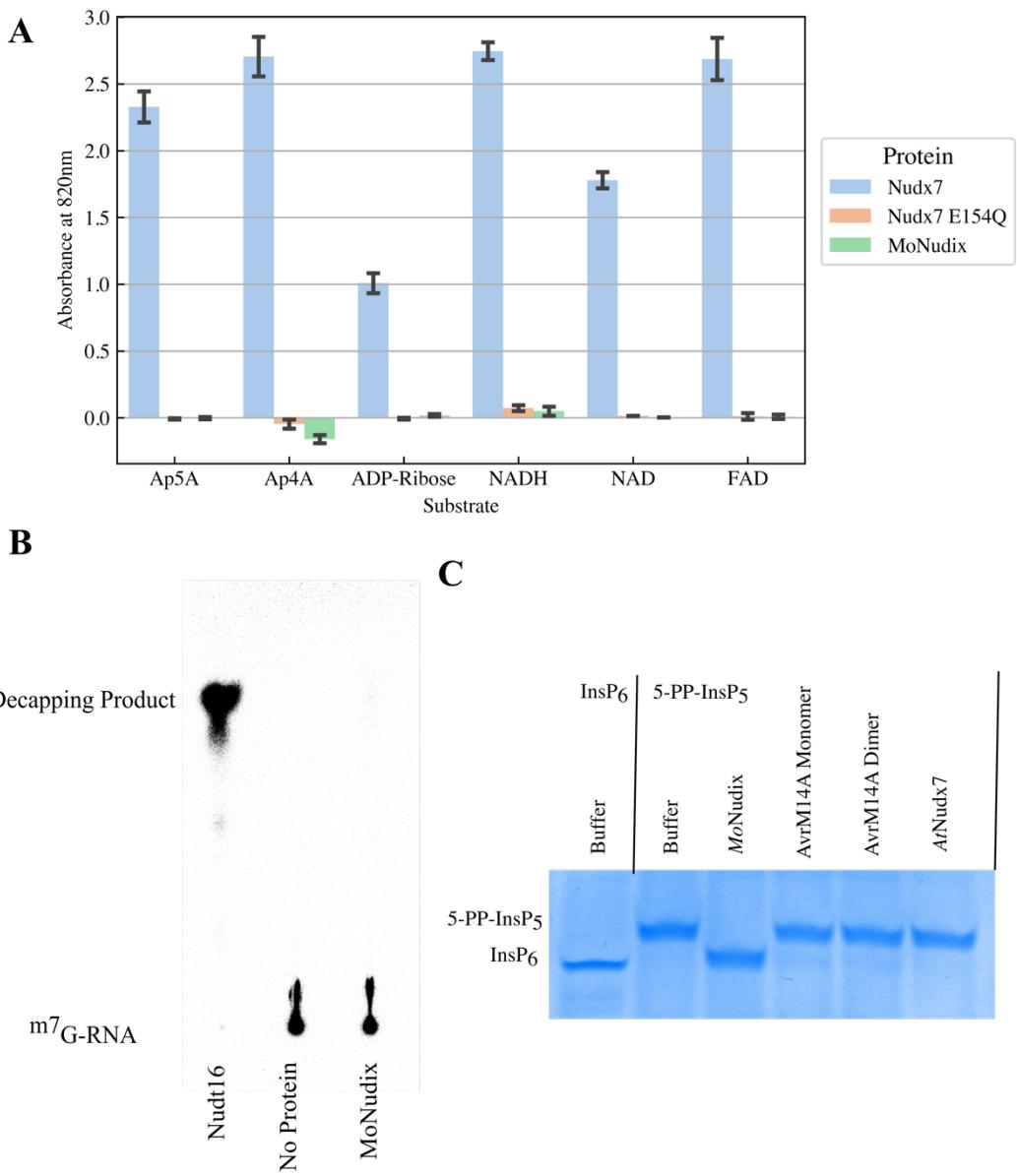


Figure S3: MoNudix does not effectively hydrolyse the substrates hydrolysed by previously characterised Nudix effectors *in vitro*. Related to Figure 1. (A) Purified recombinant proteins (*At*Nudx7, *At*Nudx7^{E154Q}, and *Mo*Nudix) were incubated with 2 mM of the indicated substrate at 37 °C for 30 minutes. Substrate hydrolysis was detected via the production of a blue-coloured phosphomolybdate complex that absorbs light with a wavelength of 820 nm. Results are presented as mean absorbance ± standard deviation (n = 3). A buffer only control without any Nudix hydrolase protein was used to blank the spectrophotometer before measurement. *At*NUDX7 and *At*NUDX7^{E154Q} were used as positive and negative controls, respectively. (B) Recombinant proteins (*Hs*Nudt16, a known mRNA decapping enzyme,¹²² and *Mo*Nudix) were incubated with $m^7Gp_{32}pp$ -RNA and the reaction products analysed by thin-layer chromatography (TLC). Capped RNA remains at the origin of the TLC plate, whereas decapping products migrate up the plate. (C) Purified recombinant protein (*Mo*Nudix, AvrM14-A monomer, AvrM14-A homodimer, *At*Nudx7) at a concentration of 5 μ M or protein storage buffer was incubated with either inositol hexakisphosphate (InsP₆) or an inositol pyrophosphate (5-PP-InsP₅) (indicated along the top of the gel) for 60 minutes at 37 °C. The reaction products were separated using a polyacrylamide gel and visualised by staining

with toluidine blue. The downwards shift of the band in the *MoNudix* lane when compared to the other 5-PP-InsP₅ treatments, indicates that *MoNudix* converts 5-PP-InsP₅ to InsP₆.

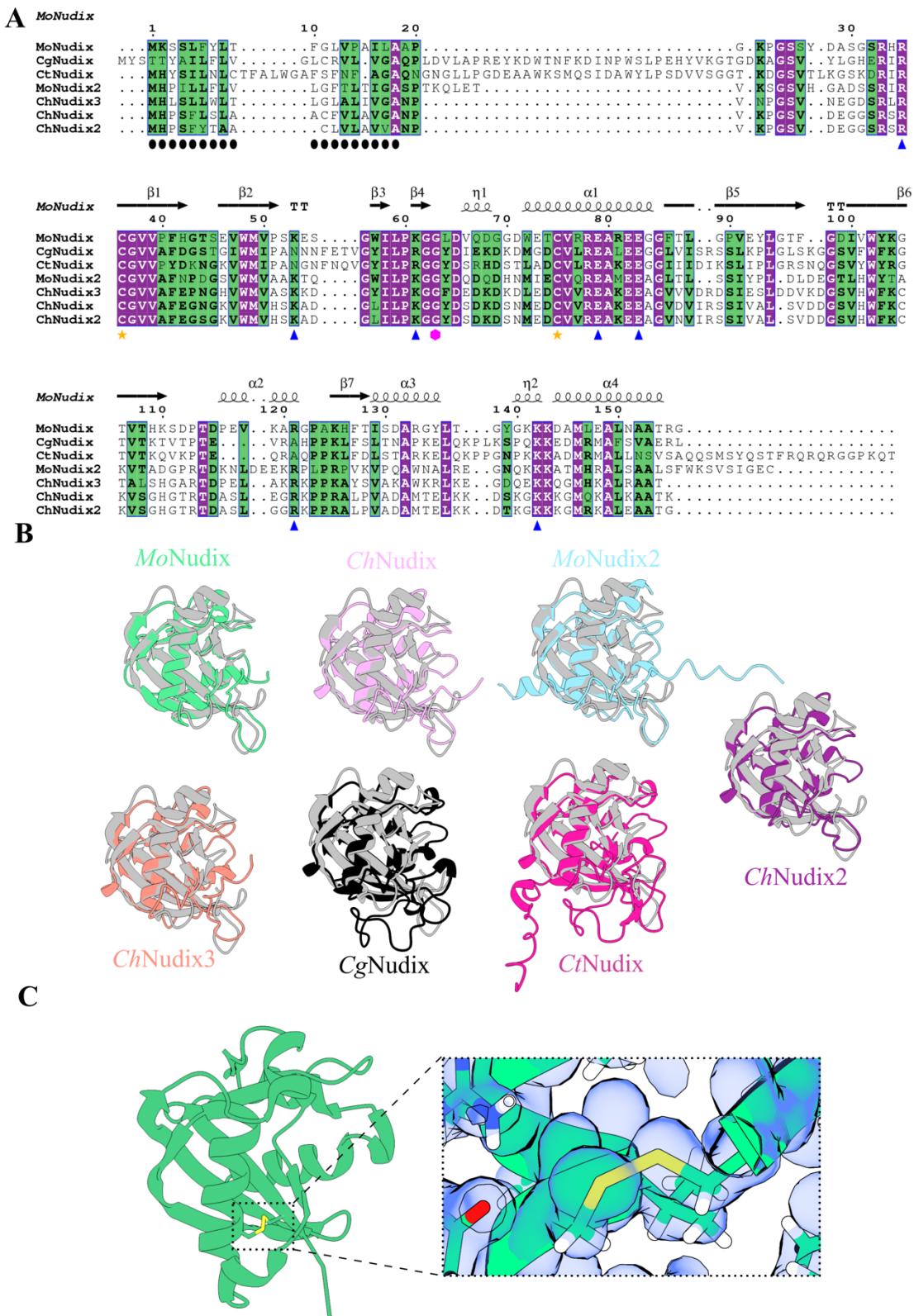


Figure S4: MoNudix and homologous effectors exhibit structural similarity to *HsDIPP1* and conservation of disulphide bonded cysteines as well as the predicted signal peptide and amino acids likely involved in PP-InsP binding and hydrolysis. Related to Figure 2.

coloured purple, conserved substitutions are coloured green, non-conserved residues are white. The predicted signal peptide region from *MoNudix* is indicated by black circles, the cysteines involved in disulphide bond formation are indicated by stars, the conserved glycine at position +1 in the Nudix box by a pink hexagon, and the amino acids likely involved in PP-InsP binding and hydrolysis in *MoNudix* are indicated by blue triangles. Above the sequence alignment is the secondary structure of *MoNudix* (black arrows indicating β -strands, squiggles indicating helices, and the TT symbol indicating β -turns. (B) Ribbon diagrams of predicted AlphaFold structures of *Magnaporthe* and *Colletotrichum* Nudix effectors, aligned with *HsDIPP1* (6WO7) in grey. (C) A ribbon diagram of *MoNudix* (PDB ID: 8SX5) with the disulphide bond show as a stick model. Inset is a zoomed view of the disulphide bond with the electron density maps displayed as a transparent blue surface contoured at a rmsd of 1.5 Å and amino acid side chains displayed as stick models, demonstrating the clear covalent bond between the cysteines.

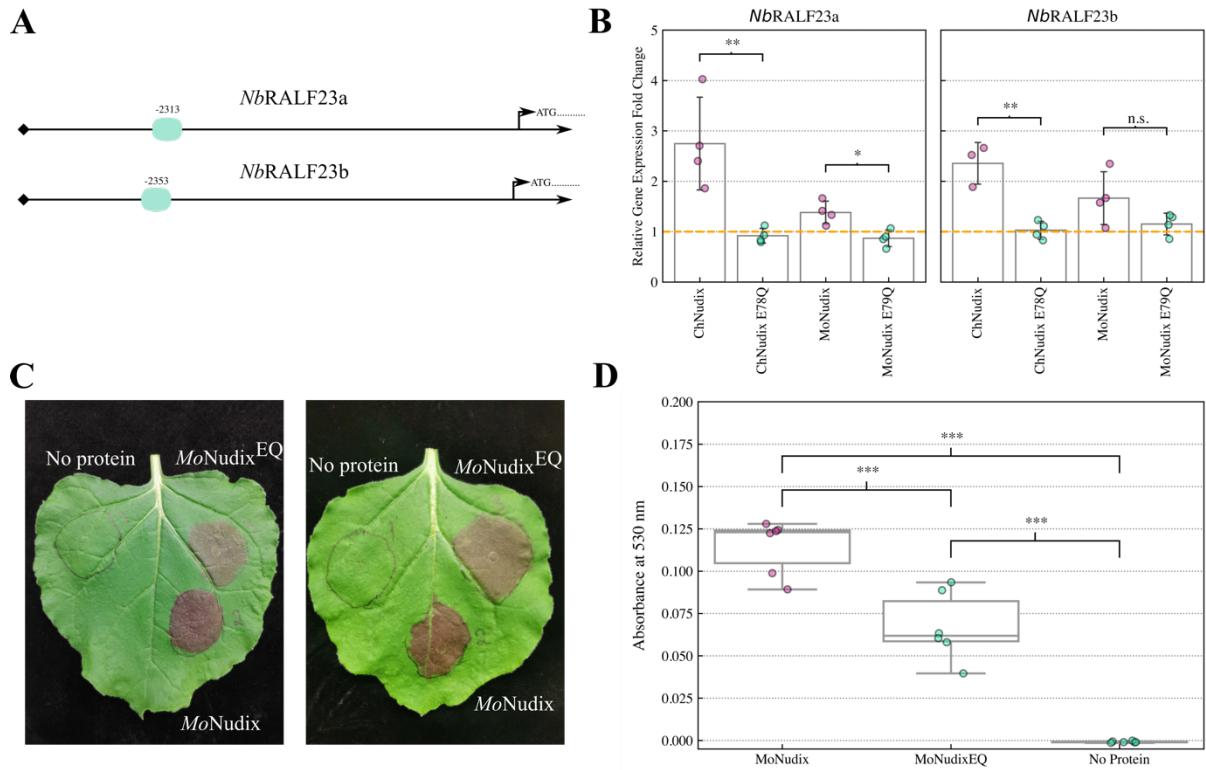


Figure S5: The enzymatic activity of the *Magnaporthe* and *Colletotrichum* Nudix hydrolase effectors induce phosphate starvation responses in *Nicotiana benthamiana*. Related to Figure 3. (A) A diagram of the 3 kb region upstream of two *N. benthamiana* RALF genes with all P1BS elements indicated by a green circle. (B) RT-qPCR was completed on RNA extracted from *N. benthamiana* leaf tissue expressing either MoNudix, ChNudix, MoNudix^{E79Q}, ChNudix^{E78Q}, or leaf tissue transformed with an empty vector control. Values represent mean \pm SD across three or four biological replicates with all individual data points shown as dots; *** p-value ≤ 0.001 (independent samples t-test). The relative gene expression fold change was calculated using the $2-\Delta\Delta Ct$ method via comparison to the vector-only control treatment. The geometric mean of two reference genes was used for normalisation (*NbeIF1a* and *NbUbe35*). The yellow dotted line indicates the relative expression level in the vector-only control treatment. (C) Representative leaf images demonstrating production of the red betalain pigment in leaf tissue co-transformed with the PEPC:RUBY promoter/reporter gene and a Nudix effector, or no effector, as labelled. (D) The absorbance at 530 nm of extracts from *N. benthamiana* leaf tissue co-transformed with the PEPC:RUBY promoter/reporter with the wild-type MoNudix effector, the corresponding Nudix box mutant protein, or no effector (as indicated). There were 6 biological replicates for each treatment, as indicated by the dots on top overlaying the boxplots. In the boxplots, the horizontal line in the middle of the box represents the median value, the box represents the interquartile range (IQR), and the whiskers extend to 1.5 x IQR. To determine whether there was a significant difference between the treatments, a one-way ANOVA was completed followed by Tukey's post-hoc test; *** adjusted p-value ≤ 0.001 .

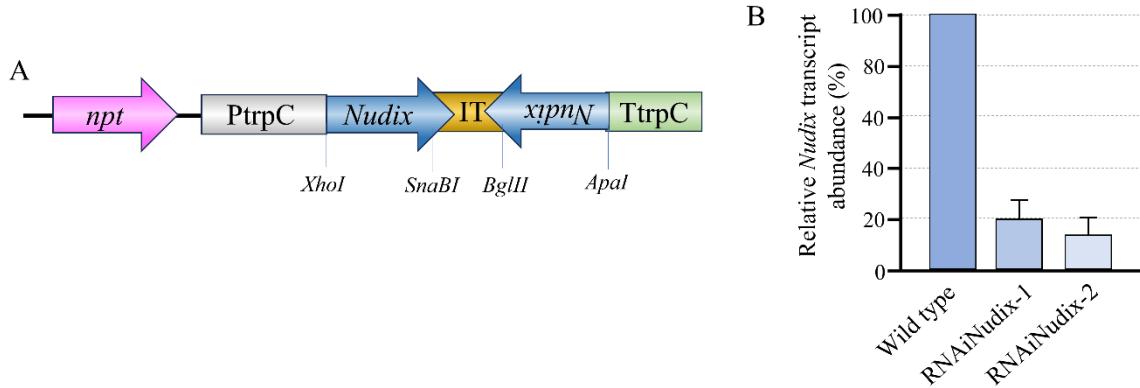


Figure S6: Silencing of *MoNudix* in *Magnaporthe oryzae*. Related to Figure 3. (A) RNAi cassette transformed into *M. oryzae*. Consisting of the TrpC promoter (P_{trpC}) from *A. nidulans*, followed by 300-bp of the sense and antisense of *MoNudix* sequence (*Nudix*) separated by the Intron2 from the Cutinase2 of *M. oryzae* (IT), followed by the TrpC terminator (T_{trpC}). The neomycin phosphotransferase (npt) resistance cassette was used as a resistance marker. Not to scale. (B) Relative transcript abundance of *M. oryzae* *MoNudix* in infected leaf sheaths at 28 hpi. Three biological replications with 9 infected leaf sheaths each were evaluated. Error bars indicate standard deviations.

Table S1: The names, species, host plant, protein sequences, and accession IDs of identified *Magnaporthe*, *Colletotrichum*, and *Ceratocystis* Nudix effectors. Related to Figure 1.

Table S2: Primer sequences used throughout this study. Related to Figure 3, Figure 4, Figure S5 and Figure S6.

Table S3: Promoter sequences discussed in this study. Related to Figure 3 and Figure S5.

Table S4: Sequences of the purified proteins used in this study. Related to Figure 1, Figure 2, Figure S2, and Figure S3.

Table S5: Crystallography data collection and structure refinement statistics for 8SXS. Related to Figure 2 and Figure S4.

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Chapter 4 Discussion, conclusions, and future perspectives

4.1 Summary of key findings

Nudix hydrolase effectors are widespread in phytopathogens, with examples in plant infecting bacteria, oomycetes and fungi (Dong and Wang 2016). The conservation of the Nudix motif has led to suggestions that effectors within this class may have similar functions during infection. Often, researchers have compared Nudix effectors to *A. thaliana*'s Nudx7, a known negative regulator of plant immunity, speculating that these effectors may mimic Nudx7 to suppress immunity and promote infection.

In this thesis, I establish that the Nudix hydrolase effectors from phytopathogenic fungi, including species from *Melampsora*, *Colletotrichum* and *Magnaporthe* genera, target substrates distinct from those hydrolysed by Nudx7 and the Nudix effectors from bacteria and oomycetes. Notably, the fungal effectors exhibit greater substrate specificity than previously characterised Nudix effectors and can be divided into two sequence-related groups with differing substrate specificity. Despite hydrolysing different substrates, both groups of fungal Nudix effectors can suppress plant immune responses.

In chapter 2, I determined that AvrM14 from *M. lini* is a highly specific Nudix hydrolase enzyme capable of removing the protective 5' cap from mRNA transcripts. Intriguingly, AvrM14 homodimerisation alters the pyrophosphate bond hydrolysed by the enzyme, suggesting unique modes of substrate binding in the monomer and homodimer. I determined that four homologues of AvrM14 from other *Melampsora* species also decap mRNA, establishing the conservation of enzymatic activity throughout the evolution of this effector family. AvrM14 expression in flax results in M1-triggered HR suppression and alters the abundance of certain circadian-rhythm associated mRNA transcripts. Collectively, the findings from chapter 2 support a model where the *Melampsora* spp. Nudix hydrolase effectors promote infection by decapping plant mRNA to suppress immune responses.

Chapter 3 focuses on characterising *MoNudix* from *M. oryzae*. Unlike AvrM14, *MoNudix* displayed no mRNA decapping activity *in vitro*. However, *MoNudix* and sequence-related effectors from *Colletotrichum* spp. effectively hydrolysed IPs. Despite low sequence similarity, *MoNudix*'s crystal structure closely resembled DIPP1, a well-characterised IP hydrolase from *Homo sapiens*. When expressed in *N. benthamiana*, the Nudix hydrolase activity of *MoNudix* and homologous effectors induced phosphate starvation responses, consistent with reduced IP levels. The starvation responses coincided with ROS burst suppression in *N. benthamiana*

leaves. I confirmed the critical role of two binding site lysines via mutagenesis to glutamate, which reduced InsP_6 binding affinity, IP hydrolase activity, and phosphate starvation induction when expressed in *N. benthamiana*. Our collaborators then determined that *MoNudix* is important for *M. oryzae* virulence on rice by reducing its gene expression using RNAi. Additionally, their live-cell imaging of *M. oryzae* expressing mRFP-tagged *MoNudix* indicates that the effector functions within rice cells during infection.

4.2 Enzymatic effectors from pathogenic fungi

Only a few fungal effectors are predicted to have enzymatic activity. Of the enzymatic effectors studied to date, most are plant cell-wall degrading/modifying enzymes, including cellulases, glucanases, hemicellulases, xylanases, and pectin lyases (reviewed in (Kubicek *et al.*, 2014)). Another class of enzymatic fungal effectors are chitinases; these are conserved in diverse pathogenic fungi and typically function by degrading chitin oligomers to evade detection by plant chitin receptors (Mart Nez-Cruz *et al.*, 2021). I demonstrate that AvrM14 and *MoNudix* enzymatic activity is essential for their suppression of the MAMP-triggered ROS burst. This contrasts with chitinase effectors, where enzymatic activity is not always required to suppress immunity; non-catalytic chitinase-like effectors retain chitin-binding properties and can block chitin recognition (Fiorin *et al.*, 2018).

The chitin and plant cell-wall targeting effectors act in the apoplast, whereas previous research on AvrM14 (Anderson *et al.*, 2016), and the data presented on *MoNudix* localisation in Chapter 3, indicates that fungal Nudix effectors function within the host cell cytoplasm. There exist only a few examples of cytosolic fungal effectors with enzymatic activity (reviewed in (He *et al.*, 2020)). Two effectors from the pathogenic fungi *Ustilago maydis* and *Verticillium dahliae* possesses chorismate mutase and isochorismate mutase activity (Djamei *et al.*, 2011, Liu *et al.*, 2014). The substrates of both enzymes are important for the synthesis of salicylic acid (SA) and accordingly, the effectors influence SA metabolism in their host plants. The Nudix hydrolase effectors, as well as the chorismate and isochorismate mutases, were originally predicted to be enzymes by identifying conserved amino acid sequence motifs. In contrast, the chitinase effectors were predicted from computational protein structure modelling (Mart Nez-Cruz *et al.*, 2021).

Recently, Seong and Krasileva (2021) utilised computational protein structure modelling and identified multiple effectors with predicted similarity to a diverse range of enzymes (Seong and Krasileva 2021). Some of these lack amino acids essential for catalysis, suggesting that

they may contribute to pathogenicity through non-enzymatic means, such as sequestration of target molecules (Seong and Krasileva 2021). I anticipate that the rapid advances in protein structure prediction will accelerate the identification of fungal effectors with enzymatic activity, leading to a greater understanding of fungal pathogenesis in plants.

4.3 Substrate selectivity mechanisms in Nudix hydrolases

The Nudix motif provides a versatile scaffold which facilitates the hydrolysis of various compounds with a pyrophosphate. This motif typically forms a loop-helix-loop structure, where amino acids within the conserved sequence enable pyrophosphate binding mediated by divalent cations. The Nudix motif does not interact other regions of the substrate; Nudix hydrolase substrate specificity therefore arises from structural features outside of the Nudix motif.

In a limited subset of Nudix hydrolases, conserved amino acid sequence motifs external to the Nudix box refine substrate specificity. For example, NAD⁺/NADH and NAD-capped RNA targeting Nudix hydrolases from evolutionary diverse organisms, including *E. coli*, *S. cerevisiae*, and *H. sapiens*, all possess an SQXWPXP(X)S motif on the C-terminal side of the Nudix box (Dunn *et al.*, 1999). Structural analysis of nicotinamide-bound NudC from *E. coli* revealed that the conserved tryptophan forms a π - π stacking interaction with the nicotinamide sugar ring and the final serine is involved in hydrogen bonding with a phosphate (Höfer *et al.*, 2016, Zhang *et al.*, 2016). Π -stacking via aromatic amino acids is a common mechanism utilised by Nudix hydrolases that bind to substrates with ring structures (Wakamatsu *et al.*, 2008, Svensson *et al.*, 2011, Tang *et al.*, 2015, Sharma *et al.*, 2020), notably both AvrM14 and *MoNudix* lack aromatic amino acids in and around the substrate binding site and demonstrate no conserved sequence motifs external to the Nudix box.

NudC's conserved tryptophan is at the beginning of a loop region that overhangs the substrate binding site (Höfer *et al.*, 2016, Zhang *et al.*, 2016). A meta-analysis of substrate-bound Nudix hydrolase structures demonstrates that amino acids within this loop are regularly involved in substrate interactions, and therefore often play a role in substrate selectivity (Srouji *et al.*, 2017). Srouji *et al.*, (2017) named this the X-loop, as it often binds to the X-moiety in Nudix (**N**ucleoside **d**iphosphate linked to moiety-**X**) substrates. The crystal structures from chapter 2 demonstrate that the AvrM14-A X-loop is from proline-103 to valine-111 (PGSKDTQRV), with the threonine replaced by proline in AvrM14-B. It is unlikely that the X-loop plays a critical role in AvrM14 substrate binding, as this region demonstrates no discernible

conservation across AvrM14 homologs which all retain mRNA decapping activity. Similarly, the X-loop from *MoNudix* and homologs is unlikely to contribute to IP binding. The *MoNudix* crystal structure demonstrates a very short X-loop (FGDI), which does not overhang the binding site, and the distance between the X-loop and the putative IP binding site likely prevents any interactions. This contrasts with *HsDIPP1*, where the X-loop overhangs the binding site and an arginine in this loop interacts with a phosphate on the inositol ring (Zong *et al.*, 2021).

Without functional X-loops the Nudix effectors must rely on other structural features to achieve substrate selectivity. My attempts to co-crystallise AvrM14 with the mRNA cap analogue molecule ${}^{m7}Gp_5G$ to understand substrate binding were unsuccessful. Mugridge *et al.*, (2016) characterised Dcp2 mRNA-cap binding via co-crystallisation with ${}^{m7}Gp_5ppp{}^{m7}G$. Unlike ${}^{m7}Gp_5G$, this molecule is not hydrolysed during Dcp2 crystallisation, however, producing it requires specialised chemical synthesis and therefore it was not utilised in my study (Mugridge *et al.*, 2016). In the absence of a substrate bound structure or notable similarity to previously studied Nudix hydrolases, the amino acids involved in AvrM14 substrate specificity remain enigmatic. For *MoNudix*, I attempted co-crystallisation with $InsP_6$, the predicted product of 5-PP- $InsP_5$ hydrolysis. Chapter 3 demonstrates that $InsP_6$ binds to *MoNudix*, and it was required for *MoNudix* crystallisation; however, $InsP_6$ could not be unambiguously placed in the electron density maps. Despite this, the structural similarity between *MoNudix* and *HsDIPP1* enabled the identification of putative substrate binding amino acids. The importance of two lysines was confirmed for $InsP_6$ binding, 5-PP- $InsP_5$ hydrolysis, and the induction of phosphate starvation in *N. benthamiana*.

Overall, my findings indicate that while the X-loop can be a determinant of substrate specificity, its involvement in substrate binding is not an absolute requirement. As the enzymatic activity of more sequence diverse Nudix hydrolases like AvrM14 and *MoNudix* are characterised, our ability to predict substrate specificity from sequence will likely improve.

4.4 Effectors targeting RNA decapping and the roles of RNA decapping during plant immunity

The characterisation of AvrM14 presented in chapter 2 is the first evidence for an effector from a plant pathogen acting as an mRNA decapping enzyme. Decapping facilitates mRNA decay from the 5' end by exoribonucleases, suggesting that AvrM14 may accelerate host mRNA decay. AvrM14 is analogous to the mRNA decapping Nudix hydrolases encoded by certain

mammalian viruses which can promote mRNA decay in the host cell (Parrish and Moss 2007, Parrish *et al.*, 2007, Quintas *et al.*, 2017).

In addition to AvrM14, the effector PST02549 from *Puccinia striiformis* f. sp. *tritici* may also target mRNA decapping pathways to promote pathogen virulence. PST02549 localises to processing bodies (P-bodies) (Petre *et al.*, 2016), which are ribonucleoprotein particles containing non-translating mRNAs along with various proteins involved in mRNA metabolism, including mRNA decapping proteins (Sheth and Parker 2003). Furthermore, the enhancer of mRNA decapping protein (EDC) 4 from *N. benthamiana* was identified as potential interactor of PST02549 (Petre *et al.*, 2016). EDC4 regulates the activity and specificity of the mRNA decapping Nudix hydrolase Dcp2 (Chang *et al.*, 2014, Charenton *et al.*, 2016, Valkov *et al.*, 2016, Mugridge *et al.*, 2018, He *et al.*, 2022), suggesting that PST02549 may influence host mRNA decapping during infection.

mRNA decay pathways may be a valuable target for effector proteins due to their involvement in modulating plant immune responses. During PTI, the *A. thaliana* decapping activator PAT1 is phosphorylated by mitogen-activated protein kinase 4 (MPK4) and accumulates in P-bodies (Roux *et al.*, 2015), indicating that immune signalling alters PAT1 activity. Like EDC4, PAT1 can influence mRNA decapping activity and specificity (He *et al.*, 2018, He *et al.*, 2022), and the loss of PAT1 in *A. thaliana* leads to the constitutive activation of some EDS1-dependant defence responses (Roux *et al.*, 2015). mRNA decapping and decay can also regulate plant immunity by controlling NLR transcript levels (Gloggnitzer *et al.*, 2014, Jung *et al.*, 2020), and by promoting the decay of negative regulators of plant immunity during PTI (Yu *et al.*, 2019). Mounting evidence indicates that mRNA decapping is important for the proper function of the plant immune system; further study is required to determine how fungal effectors like AvrM14 and PST02549 may exploit these pathways.

4.5 The influence of phosphate homeostasis on plant-microbe interactions

The precise regulation of phosphate levels is vital for all living organisms, but it is especially crucial for plants due to the often-limited bioavailability of phosphorus in soil. In plant cells, IPs convey phosphate availability through binding to SPX domains which regulate PHRs, the master transcriptional regulators of phosphate starvation responses. The enzymes VIH1 and VIH2 are dual-function proteins with IP synthase and hydrolase domains; these are crucial for integrating phosphate availability with the regulation of IP concentration in plants (Zhu *et al.*, 2019), where phosphate inhibits the phosphatase activity of VIH1/2, while ATP levels, which are decreased during phosphate starvation, regulate the kinase activity (Zhu *et al.*, 2019).

Altering the kinetics of the kinase and phosphatase VIH1/2 domains depending on phosphate levels, ensures IP concentration accurately reflects phosphate status. My results indicate that IP hydrolysis by *MoNudix* and homologous *Colletotrichum* effectors uncouples IP levels from phosphate availability, preventing accurate phosphate sensing and initiating starvation responses.

These Nudix effectors are particularly intriguing in the context of recent research into the regulation of plant interactions with mycorrhizal fungi; multiple independent research groups have demonstrated that the infection of monocot and dicot plants with phosphate-providing mycorrhizal fungi is dependent on the IP/PHR/SPX signalling pathway (Shi *et al.*, 2021, Wang *et al.*, 2021, Das *et al.*, 2022, Liao *et al.*, 2022). My results therefore suggest that pathogenic *Magnaporthe* and *Colletotrichum* fungi use IP hydrolysing Nudix effectors to hijack a highly conserved plant signalling pathway that functions in the promotion of beneficial plant-fungal interactions.

A notable difference between the results presented in chapter 3 and previous studies on phosphate signalling in plant microbe-interactions, is that in our experiments the activation of phosphate starvation was localised. Our findings demonstrate that localised IP depletion is sufficient for PHR activation and provide further support for the essential role of IPs in plant phosphate homeostasis. Future studies could utilise localised Nudix effector expression to determine if phosphate starvation in one area of the plant is communicated to other areas with sufficient phosphate, for example by the transport of PSI miRNAs as previously suggested (Loreti and Perata 2022). The ability of the Nudix effectors to activate PSRs likely provides considerable benefits to the pathogen. The reason for this is two-fold. First, the induction of phosphate starvation suppresses plant immune responses (Castrillo *et al.*, 2017, Tang *et al.*, 2022). Second, excessive free phosphate levels, as is expected to occur in plant cells with sufficient phosphate supply undergoing a PSR, can promote fungal infection (Campos-Soriano *et al.*, 2020).

Another difference between the work described in chapter 3 and most studies of phosphate-regulated plant-microbe interactions, is that our experiments were conducted using leaf and not root tissue. The main reason for this is that rice blast is primarily a foliar pathogen, although it can infect via roots (Sesma and Osbourn 2004). Our results suggest that like findings in roots, phosphate starvation inhibits immune responses in leaves. While there are differences in gene expression changes in rice roots and shoots during phosphate starvation, comprehensive RNA-Sequencing experiments indicate that the overall changes to cellular function are similar (Secco

et al., 2013). The integration of immune signalling and phosphate status is likely important for regulating interactions with beneficial root microbes; potentially immune suppression in leaves during phosphate starvation also benefits plants by conserving resources, as immune responses are energetically costly (Huot *et al.*, 2014).

In addition to my findings, previous research suggests that other pathogens may benefit from inducing phosphate starvation during plant infection (reviewed in (Paries and Gutjahr 2023)). As more effectors are characterised, I anticipate the identification of further mechanisms by which pathogens exploit phosphate signalling pathways in plants.

4.6 Are Nudix effectors critical pathogenicity factors in *Colletotrichum*?

Characterised *Colletotrichum* species are predominantly plant pathogens. *Colletotrichum tofieldiae* is a notable exception, it promotes *A. thaliana* growth in low-phosphate environments by transferring phosphate to the plant (Hiruma *et al.*, 2016). *C. tofieldiae* and *A. thaliana* interactions fluctuate between beneficial, benign, or parasitic, and are influenced by a variety of factors including phosphate availability, plant indole glucosinolate levels, and the production of fungal secondary metabolites (Hacquard *et al.*, 2016, Hiruma *et al.*, 2016, Frerigmann *et al.*, 2021, Hiruma *et al.*, 2023). I was unable to identify Nudix effectors in *C. tofieldiae*, and their absence is likely required to ensure *C. tofieldiae* growth remains appropriately regulated by plant phosphate status.

In contrast, *C. higginsianum*, a pathogen of *A. thaliana* and other Brassicaceae, has four putative Nudix effector genes. Two copies of *ChNudix* and one of *ChNudix2* are located on a mini-chromosome, chromosome 11, while *ChNudix3* is on chromosome 9 (Dallery *et al.*, 2017). All Nudix effectors are produced early in plant infection (O'connell *et al.*, 2012). *C. higginsianum* mutants lacking chromosome 11 display normal vegetative growth and can successfully penetrate host plant cells but demonstrate inhibited disease progression, similar to the phenotypes observed in Chapter 3 when *MoNudix* was silenced in *M. oryzae* (Plaumann *et al.*, 2018). Given that chromosome 11 has only eight predicted effectors (Dallery *et al.*, 2017), it is likely that *ChNudix* and *ChNudix2* are critical to the pathogenicity of *C. higginsianum*. Hiruma *et al.*, (2023) sequenced the genome of *C. higginsianum* KHC, a non-pathogenic strain isolated from *Scopolia japonica*. This strain maintains *ChNudix3* but has lost *ChNudix2* and both copies of *ChNudix*. Hiruma *et al.*, (2023) did not include a detailed analysis of the KHC genome; however, my examination of the available data suggests that strain KHC lacks

chromosome 11, further indicating that this chromosome's genes are non-essential for fungal growth and certain plant interactions but are critical for pathogenicity.

Chapter 3 identified *ChNudix3* as the only Nudix effector unable to trigger PHR activity in *N. benthamiana*. This observation suggests that *ChNudix3* would not compensate for the absence of the other Nudix effectors in *C. higginsianum* strains without chromosome 11. While this thesis did not investigate the molecular basis for *ChNudix3*'s lack of activity, a plausible explanation may be the substitution of a potentially critical lysine amino acid. This lysine is conserved in all other examined *Magnaporthe* and *Colletotrichum* Nudix effectors (Figure 1A). The lysine may act analogously to arginine 10 in *HsDIPP1*, which is also found in a flexible loop region immediately prior to the first β -sheet and binds to the top of 5-PP-InsP₅ (Figure 1B) (Zong *et al.*, 2021).

The curious loss of active Nudix effectors in non-pathogenic *C. higginsianum* and *C. tofieldiae*, coupled with the regulation of *C. tofieldiae*-*A. thaliana* interactions by phosphate status, hints to the Nudix effectors having a key role in determining the fate of plant/*Colletotrichum* interactions.

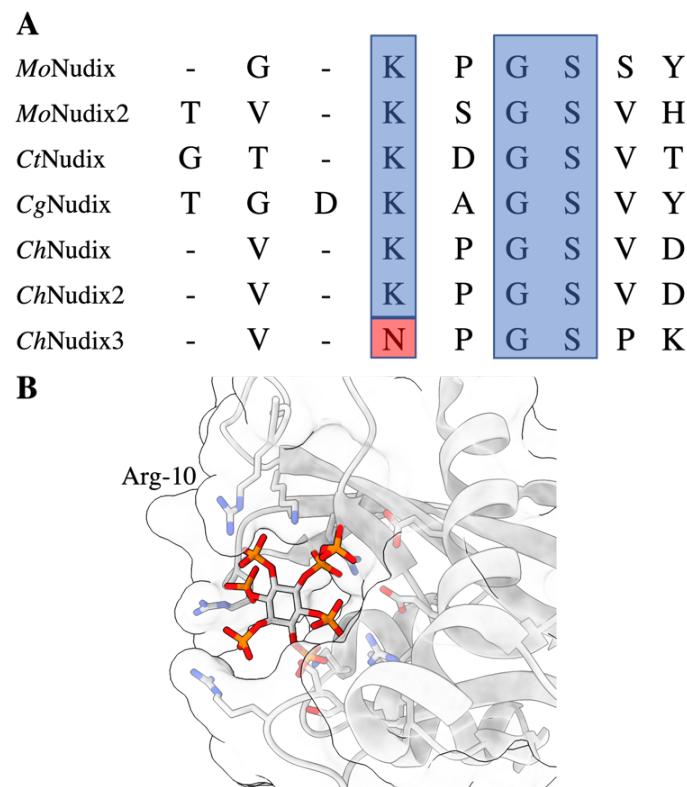


Figure 1: (A) A lysine conserved in all other tested Nudix effectors is substituted with an asparagine in *ChNudix3*. The protein sequences of the Nudix effectors were aligned using MUSCLE (Edgar 2004), with the region surrounding the lysine shown. Conserved amino acids are indicated in blue and the substituted asparagine in red. (B) The conserved lysine may act like arginine 10 from *HsDIPP1*, which is involved in 5-PP-InsP₅ binding (PDB ID: 6WO7).

4.7 Links between phosphate status and mRNA decay

AvrM14 and *MoNudix* exhibit no discernible sequence conservation outside of the Nudix motif and hydrolyse distinct substrates. However, the pathways they influence are interconnected in humans, and potentially in other organisms. This is demonstrated by studies on *HsDIPP1* and its yeast orthologue *Ddp1*, which function as both IP hydrolase and mRNA decapping enzymes (Safrany *et al.*, 1998, Safrany *et al.*, 1999, Song *et al.*, 2013, Grudzien-Nogalska *et al.*, 2016). Research by Sahu *et al.*, (2020) indicates that IPs can modulate the mRNA decapping activity of *HsDIPP1* in human cells, likely due to competition for the *HsDIPP1* active site (Sahu *et al.*, 2020). Similarly, an African Swine Fever virus Nudix hydrolase can hydrolyse both IPs and mRNA caps, and *InsP₆* can inhibit its decapping activity *in vitro* (Yang *et al.*, 2022). The benefits of IP-mediated mRNA decapping regulation are unknown, but this may represent an additional mechanism to align gene expression with phosphate availability. Whether plants possess mRNA decapping enzymes that can be inhibited by IPs, and hence if *MoNudix* promotes mRNA decapping via IP hydrolysis, remains to be determined.

4.8 Targeting Nudix effectors to achieve disease resistance

This study provides insights into fungal Nudix effectors that could inform the creation of novel R-proteins and strategies to neutralise Nudix effectors to enhance plant disease resistance. For example, Chapter 2 outlines the amino acids that enable AvrM14-B to escape M1 and M4 recognition and could therefore inform the engineering of M1 to recognise AvrM14-B. However, I propose that future research efforts should prioritise *MoNudix*, as *M. oryzae* represents a greater threat than *M. lini* to global food security (Dean *et al.*, 2012). *MoNudix* is also a promising candidate for the development of novel resistance strategies, as it is highly expressed early during infection (Yan *et al.*, 2023), and the results in Chapter 3 indicates that *MoNudix* makes a significant contribution to the virulence of the rice blast pathogen.

Recently, Kourelis *et al.*, (2023) described the engineering of plant NLRs to recognise fluorescent proteins (FP) via swapping the integrated domain in a rice CC-NLR with a nanobody that binds to the FP (Kourelis *et al.*, 2023). Nanobodies are generated by the adaptive immune system of camelids and cartilaginous fish, they are small (~15kDa) and generally very stable proteins that bind to target antigens with high affinity. *MoNudix* is straightforward to express and purify to homogeneity using an *E. coli* system, with a yield of ~10 mg of purified protein per L of bacteria culture. *MoNudix* is also exceptionally stable in solution, for example

in chapter 3 *MoNudix* was concentrated to 30 mg/mL for protein crystallisation. The highly stable, easy to purify nature of *MoNudix* would enable efficient nanobody identification and optimisation, as nanobody production methods typically require >1 mg of stable, properly folded, and highly pure protein (Pardon *et al.*, 2014). Future studies should identify nanobody binders for *MoNudix*, integrate these into the system described by Kourelis *et al.*, (2023), and generate immune receptors able to recognise *MoNudix* and confer rice blast disease resistance. To reduce the virulence of rice blast, another strategy could involve creating compounds that inhibit the enzymatic activity of the *MoNudix* effector. In appendix 1, our collaborators demonstrate that enzymatic activity is required for *MoNudix* to promote *M. oryzae* virulence. Nudix hydrolases are suitable candidates for enzyme-inhibitor design (Michel *et al.*, 2020). For example, *H. sapiens* Nudt1 plays a key role in maintaining cancer cell viability, and multiple small-molecule inhibitors of this enzyme have been developed (Gad *et al.*, 2014, Huber *et al.*, 2014, Warpman Berglund *et al.*, 2016). Likewise, inhibitors of *H. sapiens* Nudt5 and Nudt15 have been developed that bind to the active site of their targeted enzymes with low-nanomolar affinity (Page *et al.*, 2018, Zhang *et al.*, 2020). All inhibitors for Nudt1 and Nudt15 are specific to the targeted enzyme and do not target other *H. sapiens* Nudix hydrolases (Gad *et al.*, 2014, Huber *et al.*, 2014, Warpman Berglund *et al.*, 2016, Zhang *et al.*, 2020). Furthermore, no identified plant Nudix hydrolases hydrolyse IPs, it is therefore unlikely that a *MoNudix* inhibitor would interfere with the function of a plant Nudix hydrolase. Collectively, the studies with *H. sapiens* Nudix hydrolases demonstrate the potential to generate targeted small-molecule inhibitors for *MoNudix*.

However, the use of *MoNudix* small-molecule inhibitors poses a multitude of challenges, including applying the inhibitor at the correct time during the infection process, ensuring the inhibitor is taken up by the plant cell, and making enough of the inhibitor for spraying large cropping areas. Instead, the use of a protein-based inhibitor may be more feasible. A protein/peptide could be genetically encoded, eliminating the challenges described above for chemical inhibitors. Recent advances in the *de novo* design of protein binding proteins (Cao *et al.*, 2022, Bennett *et al.*, 2023, Watson *et al.*, 2023), may enable the generation of peptide/protein inhibitors for important effectors like *MoNudix* and achieve disease resistance without requiring the engineering of immunity receptors. This approach is analogous to previously identified protein-based effector inhibition systems functioning in diverse cross-kingdom interactions. For example, plant chitinases and proteinases are inhibited by fungal effectors predicted to bind to and obstruct the active site of the plant enzymes (Homma *et al.*,

2023). Another example is the toxic NAD⁺ glycohydrolase secreted by *Mycobacterium tuberculosis* during human infection, which can be inhibited by another *M. tuberculosis* protein to prevent self-poisoning (Sun *et al.*, 2015) (Figure 2). We are currently investigating both the nanobody and *de novo* protein binder strategies to target MoNudix and achieve rice blast disease resistance.

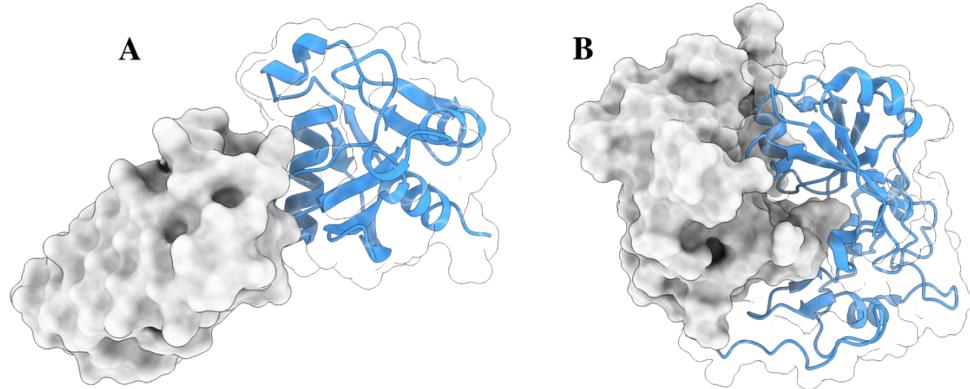


Figure 2: Enzymatic effectors could be inhibited by protein binders that obstruct the active site. (A) The crystal structure of MoNudix (transparent surface with ribbon diagram) bound to a computationally designed theoretical protein binder (solid surface). (B) The co-crystal structure of the *Mycobacterium tuberculosis* necrotizing toxin (TNT) (transparent surface with ribbon diagram) bound to its corresponding *M. tuberculosis* protein inhibitor (solid surface) (PDB ID: 4QLP).

4.9 Conclusion

This thesis has demonstrated that the predicted Nudix hydrolase effectors from phytopathogenic fungi are enzymes, characterised their substrate specificity, and identified their potential functions during pathogenesis. The work lays the foundation for further characterisation of plant mRNA decapping and IP signalling mechanisms, as well as the development of disease resistance mechanisms targeting Nudix effectors.

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