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Comparative Genomic Analysis of Effector Repertoires in Rust Fungi: Insights into Pathogenesis and Host Interactions in Wheat

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Abstract

Effector proteins play a crucial role in the interactions between rust fungal pathogens and their wheat hosts. The availability of high-throughput "omics" data has been a game-changer for the field, allowing for the identification and comparison of effectors across various rust species and strains. This study employed high-throughput "omics" data to explore the shared effector aspects of multiple rust fungi, including three wheat rust species, *Puccinia triticina*, *Puccinia striiformis*, and *Puccinia graminis*, as well as *Puccinia sorghi* (corn rust) and *Melampsora larici-populina* (poplar rust). The study employed a comprehensive bioinformatics pipeline to predict candidate secreted effector proteins (CSEPs) for each rust species, assess their subcellular localization, cluster similar effectors based on their sequence similarity, and screen their expression profiles to evaluate potential roles in pathogenesis. The study revealed diverse effectors that constitute approximately 4% of each species' proteome, with localization predictions indicating diverse targeting within host cells. Clustering of effector sequences resulted in the identification of 1,027 effector tribes and 2,186 singlets, with *P. graminis* exhibiting the highest number of singlets, suggesting accelerated evolution and adaptation to evade host defense. Conservation analysis revealed that 30 common tribes were shared among the three wheat rust species, with many also found between *P. triticina* and *P. graminis*. Expression profiling revealed differential expression during early infection, suggesting roles in pathogenesis. This study highlights the molecular diversity and adaptive strategies of rust fungi, offering insights for disease management.

Keywords – comparative analysis – disease resistance – effector proteins – pathogenesis – plant pathogen interaction – rust fungi – Wheat

Introduction

Rust fungi are economically significant pathogens, causing substantial losses in global wheat production. Wheat rust fungi, belonging to the genus *Puccinia*, are classified into three major types based on the part of the wheat plant they infect: leaf rust (*Puccinia triticina*), stem rust (*Puccinia graminis* f. sp. *tritici*), and stripe rust (*Puccinia striiformis* f. sp. *tritici*) (Bolton et al. 2008). These pathogens infect wheat leaves, stems, and grains and can spread rapidly, causing severe damage to wheat crops, including reduced yields and lower-quality grains (Singh et al. 2011, Savary et al. 2012). The adaptability of these fungi enables them to evolve into new strains capable of overcoming resistant wheat varieties. This highlights the necessity for effective management

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strategies and the development of resistant cultivars to reduce potential losses in global wheat production (Singh et al. 2011, Figueroa et al. 2018).

To mitigate the impact of wheat rust fungi, various strategies have been implemented, including the development of resistant wheat varieties, the adoption of integrated pest management practices, and the application of fungicides to control rust outbreaks (Savary et al. 2012). However, the emergence of new and more virulent races of rust fungi has posed a significant challenge to the control of these pathogens (Savary et al. 2012, Figueroa et al. 2018). The global impact of wheat rust diseases is significant and threatens global food security (Singh et al. 2006). Therefore, continued research is essential for developing genetically modified wheat to prevent and manage these pathogens, ensuring the sustainability of wheat production. However, this approach remains controversial, as some groups have raised concerns about the potential risks associated with genetically modified crops.

Comparative genomics has been instrumental in elucidating the genetic underpinnings of wheat rust fungi. By analyzing and comparing the genomes of various wheat rust fungi alongside their host genomes, researchers have identified specific genes and molecular pathways that contribute to both infection mechanisms and host resistance (Duplessis et al. 2011, Cuomo et al. 2017). This knowledge has been leveraged to develop molecular markers that enable the identification of wheat cultivars resistant to rust fungi. Furthermore, the recent accessibility of high-quality genomes for both wheat and rust fungi has provided opportunities to examine the evolutionary processes of rust fungi and their historical interactions with wheat (Zhang et al. 2022). This has revealed the complex and dynamic nature of the wheat rust pathosystem and underscored the importance of monitoring the emergence and spread of new rust strains (Garnica et al. 2013). In addition, comparative genomics has led to the discovery of new virulence factors and mechanisms of pathogenicity in rust fungi, which has opened up new avenues for the development of targeted fungicides (Duplessis et al. 2011). As technology continues to advance, comparative genomics will likely continue to play a critical role in the ongoing fight against wheat rust fungi and other plant pathogens.

Effector proteins are essential players in the complex interactions between rust fungi and their host plants, significantly influencing both virulence and resistance mechanisms. These proteins are secreted by the fungi and are designed to interact with host cells, where they can manipulate various physiological processes (Dou & Zhou 2012). By altering the host's cellular functions, effector proteins enable the pathogen to evade the plant's immune responses, promote nutrient uptake, and create a favorable environment for infection. This intricate interplay not only enhances the survival and proliferation of the rust fungi but also poses significant challenges for developing effective resistance strategies in wheat cultivation. These proteins are diverse and can have different functions, including suppressing host defense, promoting nutrient uptake, and altering the host cell structure (Selin et al. 2016). The host plant can also recognize some effectors, leading to an immune response against the pathogen. Rust fungi possess a large number of effectors, and recent studies have provided insight into their diversity, structure, and potential mechanisms (Duplessis et al. 2011, Mapuranga et al. 2022, Zhang et al. 2022). However, the precise roles of many effectors remain unknown, and further investigation is necessary to fully elucidate their functions. Understanding the function and mechanisms of effectors will provide insights into host-pathogen interactions and offer potential new strategies for disease management.

Effector prediction and classification are critical for understanding the molecular basis of pathogenicity in rust fungi and developing new strategies for disease control. By identifying effectors, we can gain insights into the mechanisms by which rust fungi infect their hosts, evade host defense, and establish infection (Garnica et al. 2013). Effector prediction and classification also allow researchers to differentiate between virulence and avirulence genes, which play key roles in host-pathogen interaction (Hogenhout et al. 2009). These predictions facilitate the identification of new effector proteins that are crucial for the pathogenicity of the rust fungi, which can be used as targets to develop effective fungicides and genetically modified crops with enhanced resistance to rust fungi (Garnica et al. 2013, De Guillen et al. 2019). Thus, effector prediction and classification

have immense potential to contribute to the development of sustainable and effective strategies for the control of rust diseases.

Effector prediction has already led to significant breakthroughs in our understanding of rust pathogenicity. For instance, researchers have discovered that the effector proteins of *P. striiformis* infecting wheat play critical roles in the preliminary stages of host infection by suppressing host immune responses (Wu et al. 2022). This finding led to the identification of potential targets for disease control and facilitated the development of new strategies for managing rust diseases. Similarly, effector prediction studies have identified key molecular targets in other rust fungi, which have helped to unravel the complex mechanisms of rust pathogenicity (Zhao et al. 2020). The identification and characterization of effectors has thus become a crucial step in the development of novel and effective approaches for the control of rust fungi, and a powerful tool for improving crop yield and food security.

Effector proteins produced by rust fungi can be broadly classified into two categories: general effectors and species-specific effectors. General effectors are secreted by rust fungi infecting multiple plant species and targeting conserved components of the plant's immune system. By disrupting the plant's defense responses, general effectors allow the rust fungi to colonize a wide range of plant hosts (Tao et al. 2017). In contrast, species-specific effectors are produced by rust fungi adapted to a particular host species. These effectors are designed to target unique features of the plant's immune system that are specific to the host species. Thus, species-specific effectors can enhance the ability of rust fungi to infect and colonize the particular host plant (Tao et al. 2017, Dos Santos et al. 2021), making them important determinants of pathogenicity. A deep understanding of the classification and function of effectors can inform the development of more effective and targeted strategies for controlling rust diseases in agriculture.

Comparative analysis of the effectoromes across various rust fungi is a critical strategy for elucidating the evolutionary relationships among different rust species and for identifying shared mechanisms of pathogenicity. This approach enhances our understanding of how these pathogens have adapted to their hosts and may reveal targets for developing more effective resistance strategies in crop management. However, this requires a comprehensive understanding of multiple rust fungal effectoromes (Nandety et al. 2022). Although there has been significant progress in characterizing the effectorome of several rust fungi, many remain unexplored (Garnica et al. 2013). By comparing effector profiles, we can gain insights into the evolutionary relationships between different rust fungi and identify conserved effectors that may be critical for pathogenicity. These insights can be used to develop new strategies for disease control and to design crops with improved resistance to rust fungi.

Recently, the sequence read archive (SRA) has advanced our understanding of genetic diversity among various races of rust fungi, particularly through the application of RNA sequencing (RNA-Seq) experiments. By providing access to a vast repository of sequencing data, the SRA facilitates comprehensive analyses of gene expression profiles during rust infections. This enables researchers to identify key virulence factors and resistance mechanisms across different rust species. For instance, the integration of RNA-Seq datasets allows for simultaneous examination of gene expression dynamics over time, revealing insights into how specific genes are activated in response to host interactions (Kumar et al. 2020, Adams et al. 2021, Ji et al. 2022, Liu et al. 2022, Wu et al. 2023, Sánchez-Vallet et al. 2018). The ability to access and analyze these extensive datasets significantly enriches our understanding of host-pathogen interactions and fosters innovative approaches to managing rust diseases in agricultural systems.

Our study focused on a comprehensive analysis of the secretome of wheat rust fungi, with particular attention to their effector proteins. Additionally, we assessed the expression of these effector proteins in infected wheat tissue, offering insights into their role in rust pathogenesis. Our findings shed light on the intricate nature of rust pathogens, identifying key proteins critical for their pathogenicity.

Materials & Methods

Data collection

To conduct a comprehensive comparative analysis of rust candidate effectors, total protein datasets generated from five rust fungi were collected from the public repository of FTP genomic data maintained by the National Centre for Biotechnology Information (NCBI). The total protein datasets used in this investigation involved *Puccinia triticina* Pt_BBBD race 1 (GCA_000151525.2), *Puccinia striiformis* Pst_93-210 (GCA_002920065.1) and Pst_93TX-2 (GCA_002920205.1), *Puccinia graminis* Pgt_210 (GCA_008522505.1) and Pgt_CRL 75-36-700-3 (GCA_000149925.1), *Puccinia sorghi* Ps_RO10H11247 (GCA_001263375.1), and *Melampsora larici-populina* Mlp_98AG31 (GCA_000204055.1). All data were accessed on 1st June 2023 listed in Table (1).

Table 1. Rust fungal genome datasets collected to predict their secretomes.

Fungal Agent	Strain	Rust Disease	GCA Data
Puccinia triticina	*Pt*_BBBD, race 1	Leaf rust	GCA_000151525.2
Puccinia striiformis	*Pst*_93-210	Yellow rust	GCA_002920065.1
Puccinia striiformis	*Pst*_93TX-2	Yellow rust	GCA_002920205.1
Puccinia graminis	*Pgt*_210	Stem rust	GCA_008522505.1
Puccinia graminis	*Pgt*_CRL75-36-700-3	Stem rust	GCA_000149925.1
Puccinia sorghi	*Ps*_RO10H11247	Common rust of maize	GCA_001263375.1
Melampsora larici-populina	*Mlp*_98AG31	Rust disease in poplar	GCA_000204055.1

Candidate effector protein prediction pipeline

The identification of rust effector proteins was performed using bioinformatics-based prediction tools to analyze the fungal secretome. First, the presence of signal peptides, a hallmark of secreted proteins, was predicted using SignalP (http://cbs.dtu.dk/services/SignalP/, (Almagro Armenteroset et al. 2019) and Phobius (https://phobius.sbc.su.se/, (Käll et al. 2007). The overlapping sequences from the former step were subsequently submitted for transmembrane prediction, a feature that facilitates the anchoring of some effector proteins to the host cell membrane, using both Phobius and TMHMM (http://cbs.dtu.dk/services/TMHMM/, (Krogh et al. 2001). Proteins with zero transmembrane structures (PredHel) were selected. Only short proteins ≤ 300 amino acids (aa) were chosen for further analysis.

EffectorP (https://effectorp.csiro.au/, (Sperschneider et al. 2016), the machine learning method that specializes in fungal effector prediction in secretomes, was then utilized. This method employs several sequence-based features, such as amino acid composition, physicochemical properties, and motif occurrence, to determine the probability of a given protein being an effector. The outputs of this method were then used to prioritize potential effector proteins for further analysis.

To determine the subcellular localization(s) of the predicted effector proteins, we employed DeepLoc 2.0 (https://services.healthtech.dtu.dk/services/DeepLoc-2.0/, (Almagro Armenteroset et al. 2017). DeepLoc 2.0 is a multi-label predictor tool that can differentiate between ten different subcellular localizations, including the nucleus, cytoplasm, extracellular space, mitochondria, cell membrane, endoplasmic reticulum, chloroplast, Golgi apparatus, lysosome/vacuole, and peroxisome.

Clustering candidate effector proteins

To classify predicted effectors, we employed the CD-HIT algorithm (Fu et al. 2012), which groups all proteins identified as effectors collected from all investigated rust fungi based on their

sequence similarity using three cut-offs (50%, 60%, and 70%). This allowed us to group the effectors into tribes (protein families).

Annotation of candidate effectors' putative functions

To identify the function of effector proteins from rust fungi, we employed domain analysis tools, including Pfam (Finn et al. 2013). This tool allowed for the identification of conserved domains and motifs within the protein sequences, providing critical insights into their potential function and interaction partners. To complement protein analysis, we utilized the functional annotation tool Blast2GO (Conesa et al. 2005) to assign Gene Ontology (GO) terms to each identified effector.

RNA-seq data collection

Public RNA sequencing (RNA-seq) data were collected from the Sequence Read Archive (SRA) within GenBank to facilitate a comparative analysis of gene expression variations in wheat rust fungi. This dataset provides raw sequence data essential for examining the transcriptomic profiles of different rust species. We selected three RNA-seq experiments from SRA to analyze the expression of three wheat rust fungal effectors during leaf rust infection (Table 2). The libraries encompass two distinct time points of infection: 0 hours post-infection (hpi) and 24 hpi. The first dataset consisted of RNA-seq data from *T. aestivum* cultivar Toropi infected with *P. triticina* race MDT-MR (BioProject: PRJEB41456, released on November 22, 2021). The second dataset involved RNA-seq data from *T. aestivum* cultivar Canthatch K infected with *P. graminis* race QTHJC (BioProject: PRJNA401266, released on September 3, 2017). The third dataset comprised RNA-seq data from *T. aestivum* cultivar Mingxian 169 infected with *P. striformis* race CYR32 (BioProject: PRJNA637808, released on January 21, 2021). All selected datasets from the SRA were generated using paired-end Illumina sequencing on the HiSeq 2000 and 4000 platforms.

Table 2. Libraries used for studying relative expression level of wheat rust effectors.

BioProject	Pathogen	Race	Wheat cultivar	Wheat age	Stage
PRJEB41456	*P. triticina*	MDT-MR	Toropi	60 days	0 hpi
PRJEB41456	*P. triticina*	MDT-MR	Toropi	60 days	0 hpi
PRJEB41456	*P. triticina*	MDT-MR	Toropi	60 days	0 hpi
PRJEB41456	*P. triticina*	MDT-MR	Toropi	60 days	24 hpi
PRJEB41456	*P. triticina*	MDT-MR	Toropi	60 days	24 hpi
PRJEB41456	*P. triticina*	MDT-MR	Toropi	60 days	24 hpi
PRJNA401266	*P. graminis*	QTHJC	Canthatch K	21 days	0 hpi
PRJNA401266	*P. graminis*	QTHJC	Canthatch K	21 days	0 hpi
PRJNA401266	*P. graminis*	QTHJC	Canthatch K	21 days	0 hpi
PRJNA401266	*P. graminis*	QTHJC	Canthatch K	21 days	24 hpi
PRJNA401266	*P. graminis*	QTHJC	Canthatch K	21 days	24 hpi
PRJNA401266	*P. graminis*	QTHJC	Canthatch K	21 days	24 hpi
PRJNA637808	*P. striiformis*	CYR32	Mingxian 169	21 days	0 hpi
PRJNA637808	*P. striiformis*	CYR32	Mingxian 169	21 days	0 hpi
PRJNA637808	*P. striiformis*	CYR32	Mingxian 169	21 days	0 hpi
PRJNA637808	*P. striiformis*	CYR32	Mingxian 169	21 days	24 hpi
PRJNA637808	*P. striiformis*	CYR32	Mingxian 169	21 days	24 hpi
PRJNA637808	*P. striiformis*	CYR32	Mingxian 169	21 days	24 hpi

Data quality control

To ensure precision, a comprehensive quality control process was executed. The FastQC toolkit was utilized to evaluate the quality of the raw sequencing data, facilitating the identification of suboptimal sequences, overrepresented sequences, and adapter contamination (Andrews et al. 2010). Furthermore, the Trimmomatic tool was applied to trim and filter out low-quality sequences and contaminants, thereby enhancing the overall data integrity (Bolger et al. 2014).

Alignment and expression normalization

To facilitate the alignment of high-quality short reads to the three-wheat rust fungal effector reference sequences, the Bowtie mapping program was utilized (Langmead & Salzbeng 2012). This step involved mapping the filtered reads to the reference cDNAs, ensuring accurate positioning and alignment. Following this, the gene expression levels were quantified and presented as Transcripts Per Million reads of library (TPM) using the StringTie software (Pertea et al. 2015). This quantification provided a normalized measure of gene expression, enabling comparative analysis across different samples. Subsequently, the TPM values were subjected to differential expression analysis using DESeq2, a widely recognized and robust tool for identifying statistically significant changes in gene expression levels (Love et al. 2014). This comprehensive approach ensured a thorough and reliable assessment of gene expression dynamics.

Statistical analysis and heatmap generation

The statistical analysis and heatmap figures in this investigation were produced using R, version 4.3.0. Heatmaps were created with the pheatmap v1.0.12 R package, facilitating the visualization of complex gene expression patterns (https://cran.rproject.org/web/packages/pheatmap/index.html, (Dessau & Pipper 2008).

Results

The rationality of selecting rust fungal strains

The availability of high-throughput "omics" data has been a game-changer for the field of fungal disease, allowing for the identification and comparison of effectors across various rust species and strains. We analyzed the total deduced proteins of the three wheat rust fungi, including some variations across strains, to gain insights into the evolution of wheat rust effectors. To gain a broader perspective on rust pathosystems, we expanded our analysis by including corn and poplar rust, which demonstrate a spectrum of dissimilarities across many species and even genera of rust fungi. The datasets encompassed Pt_BBBD race 1 of *P. triticina*, Pst_93-210 and Pst_93TX-2 races of *P. striiformis*, Pgt_210 and Pgt_CRL 75-36-700-3 races of *P. graminis*, Ps_RO10H11247 race of *P. sorghi*, and Mlp_98AG31 race of *M. larici-populina* (Table 1). The sizes of their genomes varied depending on the rust species, ranging in size from ~ 176 to ~ 77 Mb for Pgt_210 and Pst_93TX-2, which encoded 37,843 to 14,629 proteins, respectively. This collection of datasets not only facilitates a more thorough investigation of effectors, but also enhances our understanding of the mechanisms of rust pathogenicity on plants.

Rust fungal effectors' prediction

Secretome prediction relies on two fundamental principles: 1) the identification of a secretion signal and 2) the absence of transmembrane helices (Agrawal et al. 2010). Here, the candidate effectors of Pt_BBBD race 1, Pst_93-210, Pst_93TX-2, Pgt_210, Pgt_Ps_CRL 75-36-700-3, Ps_RO10H11247, and Mlp_98AG31 were predicted. A total of 15,685, 15,090, 14,629, 37,843, 15,979, 21,078, and 16,257 deduced proteins were screened, respectively. A consistent and comprehensive pipeline for the protein datasets was developed to ensure uniformity in data analysis (Fig. 1). The entire set of proteins of the seven fungal datasets were analyzed to determine the presence of signal peptides and the absence of transmembrane domains. Only short proteins with a length of 300 amino acids or less were selected for further analysis due to their potential for

secretion and their impact on plant hosts. This criterion aligns with findings from previous studies that highlight the prevalence of small secreted proteins in fungal secretomes, which are often involved in manipulating host interactions (Kim et al. 2016). Following this, we used EffectorP to analyze all proteins selected from previous screening to determine their likelihood of being effectors. The tool uses several features of the protein sequences, such as the presence of signal peptides, homology to known effectors, and the presence of certain domains, to predict whether a protein is likely to be secreted and function as an effector. This resulted in 472, 563, 581, 2286, 752, 187, and 655 candidate secreted effector proteins (CSEPs) for Pt_BBBD race 1, Pst_93-210, Pst_93TX-2, Pgt_210, Pgt_CRL 75-36-700-3, Ps_RO10H11247, and Mlp_98AG31, respectively (Fig. 1, Table 3). Of these datasets, approximately 4%, on average, of the selected deduced proteomes were predicted as effector proteins. The highest number of effector proteins was predicted in Pgt_210 (2286 proteins, 6%), while the lowest number was predicted in Ps_RO10H11247 (187 proteins, 1%).

Fig. 1 – Chart for identifying candidate rust effector proteins and their gene expression profile. The chart outlines the pipeline used to predict candidate effector proteins for various rust species and races, including Pt_BBBD race 1, Pst_93-210, Pst_93TX-2, Pgt_210, Pgt_Ps_CRL75-36-700-3,

Ps_RO10H11247, and Mlp_98AG31. The numbers outside the graph indicate the total count of proteins retrieved after each filtration step. A. The complete proteomes of the seven fungal datasets were screened to identify candidate effector proteins. B. The identification of secretion signal peptides was conducted using SignalP and Phobius, "C." while the absence of transmembrane helices was assessed through TMHMM and Phobius. D. The likelihood of each protein being an effector was evaluated using EffectorP. E. DeepLoc analysis was performed to predict the subcellular localization of rust effectors within host cells. Potential localizations include the cytoplasm (C), nucleus (N), mitochondria (Mt), cell membrane (CM), plastid (P), lysosome (Ly), endoplasmic reticulum (ER), peroxisome (Px), and Golgi apparatus (G). Effector proteins were clustered using the CD-HIT program to group related proteins based on sequence similarity. G. Identification of conserved domains and potential functional roles among different rust fungi species were assigned using BLAST2GO suite. H. Sequence Read Archive (SRA) data were utilized to assess the gene expression levels of candidate effectors.

Table 3. The counts of proteins predicted to be short effectors.

Fungal Agent	Strain	Total Protein	EffectorP ≤ 300AA	% of the Effectors
Puccinia triticina	*Pt*_BBBD, race 1	15,685	472	3
Puccinia striiformis	*Pst*_93-210	15,090	563	4
Puccinia striiformis	*Pst*_93TX-2	14,629	581	4
Puccinia graminis	*Pgt*_210	37,843	2286	6
Puccinia gramini	*Pgt*_CRL 75-36-700-3	15,979	752	5
Puccinia sorghi	*Ps*_RO10H11247	21,078	187	1
Melampsora larici-populina	*Mlp*_98AG31	16,257	655	4

Subcellular localization prediction of fungal rust effectors

Deciphering the subcellular localization of fungal rust effectors is a critical factor in uncovering their mechanisms of interaction with host cells, since it helps to determine the site of action of these effectors within the host cell. Here, we explored the predicted subcellular localization of fungal rust effectors within host cells. To achieve this, we used DeepLoc, a deep learning-based program designed for predicting protein subcellular localization (Fig. 1). DeepLoc has an advantage over other subcellular localization prediction programs due to its capability of using various features for prediction, such as sequence-based, motif-based, and homology-based features for localization (methods). Our analysis using DeepLoc showed that rust effectors, minus their signal peptides, have a diverse range of subcellular localizations. Many of them were predicted to target outside of the host cells, termed extracellular (Ex), with the number of proteins ranging from 1260 to 71 for Pgt_210 and Ps_RO10H11247, respectively. Furthermore, other effectors targeted intracellular locations within the host cell, showcasing a diverse range of subcellular localizations. The highest numbers of proteins were found in *P. gramains* (Pgt_210) including the cytoplasm (C, 335 proteins), nucleus (N, 368 P), mitochondria (Mt, 200 P), cell membrane (CM, 35 P), plastid (P, 70 P), lysosome (Ly, 13 P), endoplasmic reticulum (ER, 7 P), and peroxisome (Px, 3 P). The diverse range of subcellular localizations observed in this study highlights the complex and dynamic nature of host-pathogen interactions during fungal rust infection.

Clustering fungal rust effectors based on sequence similarity

Evolutionary relationships among rust fungal effectors are crucial for understanding fungal general strategies for controlling plant hosts. Recently, the use of clustering algorithms, such as CD-HIT, has become a popular approach for analyzing large sets of protein sequences and identifying homologous clusters (methods). Here, we used CD-HIT to group all rust effector

protein sequences resulting from all investigated rust proteins at three different cut-offs (50%, 60%, and 70%), resulting in no differences in the clustering patterns at these three cut-offs (Fig. 2A). The number of clusters resulting from the grouping was found to be contingent on the chosen sequence similarity cut-off. Specifically, the number of clusters ranged from 3228 (31%) to 3723 (35%) when using the similarity cut-off of 50% and 70%, respectively.

Furthermore, quantifying the distribution of proteins targeted to various host cell subcellular localizations and organelles (C, N, Mt, CM, P, Ly, ER, Px, and G) using the three specified cut-off criteria indicated no significant differences among them (Fig. 2B). For proteins targeted outside of the host cells (probably apoplast candidate effectors), there was a range of approximately 337 clusters between the 50% and 70% cut-offs, so it was reasonable to choose a lower cut-off value like 50% for clustering rust effectors. This is because a lower cut-off value allowed us to relate sequences across various species and even genera. The selected cut-off (50%) resulted in 1460, 589, 621, 356, 61, 106, 9, 16, 9, and 1 protein clusters that were predicted to localize in Ex, C, N, Mt, CM, P, Ly, ER, Px, and G, respectively.

The clustering also yielded a total of 2186 unique proteins, known as “singlets”, and 1027 tribes. The repertoire of singlet effectors was observed to be large, with a molecular weight ranging from 34.38 to 3.49 kDa for singlet_1552 and singlet_258, respectively. For the tribes, tribe_1 was found to have the highest number of members, consisting of 22 members, shared among the three rust species that infect wheat. The protein length distribution across all tribes was observed to range from 300 aa for a protein with GenBank accession ID GB: KAA1101658.1 in tribe_848 to 29 aa for GB: POV95005.1 protein in tribe_274. The molecular weight of the candidate effector proteins showed significant variability, ranging from 34.18 kDa to 3 kDa, with the GB: KAA1084811.1 protein in tribe_726 having the highest molecular weight and the GB: POV95005.1 protein in tribe_274 exhibiting the lowest molecular weight. Furthermore, the average number of cysteine residues was found to be ranging from 25 cysteines in the cysteine-rich protein with GB: KAA1112910.1 in tribe_170 to zero cysteine in several proteins. This diversity in effector protein structure has been observed in other plant-pathogen interactions and is thought to contribute to the ability of pathogens to overcome host defenses. Our dataset reveals diverse structural and physical characteristics in the context of host-pathogen interactions.

Accelerated evolution of rust fungal effectors

Rust effector proteins have evolved rapidly, enabling these fungi to swiftly adapt and overcome plant immune defenses. Here, the balance between singlets and species-specific tribes of rust effectors appears to be skewed heavily towards singlets. The majority of candidate effector proteins tended to be singlets, unique to a particular species or strain, rather than part of conserved tribes (Fig. 3A). While singlets appeared to be more abundant than tribes within species, the extent of this difference varied depending on the species-specific effectors. The number of singlets varied across selected rust fungi. For instance, the effectorome of the pathogenic fungus *P. graminis* (Pgt_210) had 689 singlets, while *P. sorghi* had 142 singlets. The high degree of singlet effector divergence observed in some rust effectoromes reflects the ongoing arms race between the pathogen and its host, emphasizing the importance of effector diversity and rapid evolution in the success of rust pathogens as plant pathogens. Similarly, the number of tribes unique to each species also varied, with Pgt having 607 specific tribes and Ps having five specific tribes.

Exploring the conservation rust fungal effectors

The identification of conserved effector proteins provides a valuable resource for the development of effective control strategies against rust diseases. Comparative analysis of wheat rust effector proteins revealed the existence of conserved tribes that were shared between multiple species (Fig. 3B). One interesting finding from this comparative analysis of wheat rust effectors was the identification of tribes that were conserved across the three species of *Puccinia* that specifically infect wheat. Comparative analysis of wheat rust effector proteins revealed the existence of conserved tribes shared between multiple species. For the three wheat rust species, the

analysis revealed the existence of 30 common tribes (Fig. 3B). Interestingly, the analysis revealed that 18 of the 30 tribes were predicted to have functions outside of wheat cells, yet their specific roles remain unidentified. Additionally, among the wheat common tribes, six tribes were located in the cytoplasm, four in the nucleus, and two in the cell membrane. While many of these tribes were of unknown function, some of them were found to have functional annotations that provided insights into their potential roles in the pathogenicity of the wheat rust pathogen. For instance, two groups of proteins, identified as thioredoxin-like proteins (TLPs) and chorismate mutases (CMs), were found to belong to the cytoplasmic tribes. Tribe_785 and Tribe_990 were identified as proteasome subunit beta type-2 (PSMB2) and cupredoxin domain-containing proteins, respectively. Similarities were also detected between pairs of wheat rust fungi. Pt and Pgt have 47 tribes in common; Pgt and Pst share 40 tribes, while only 13 tribes were found to be shared by Pt and Pst. The greater number of shared tribes between Pt and Pgt indicates a closer evolutionary relationship, as these two rust fungi share significantly more tribes than the other pairs.

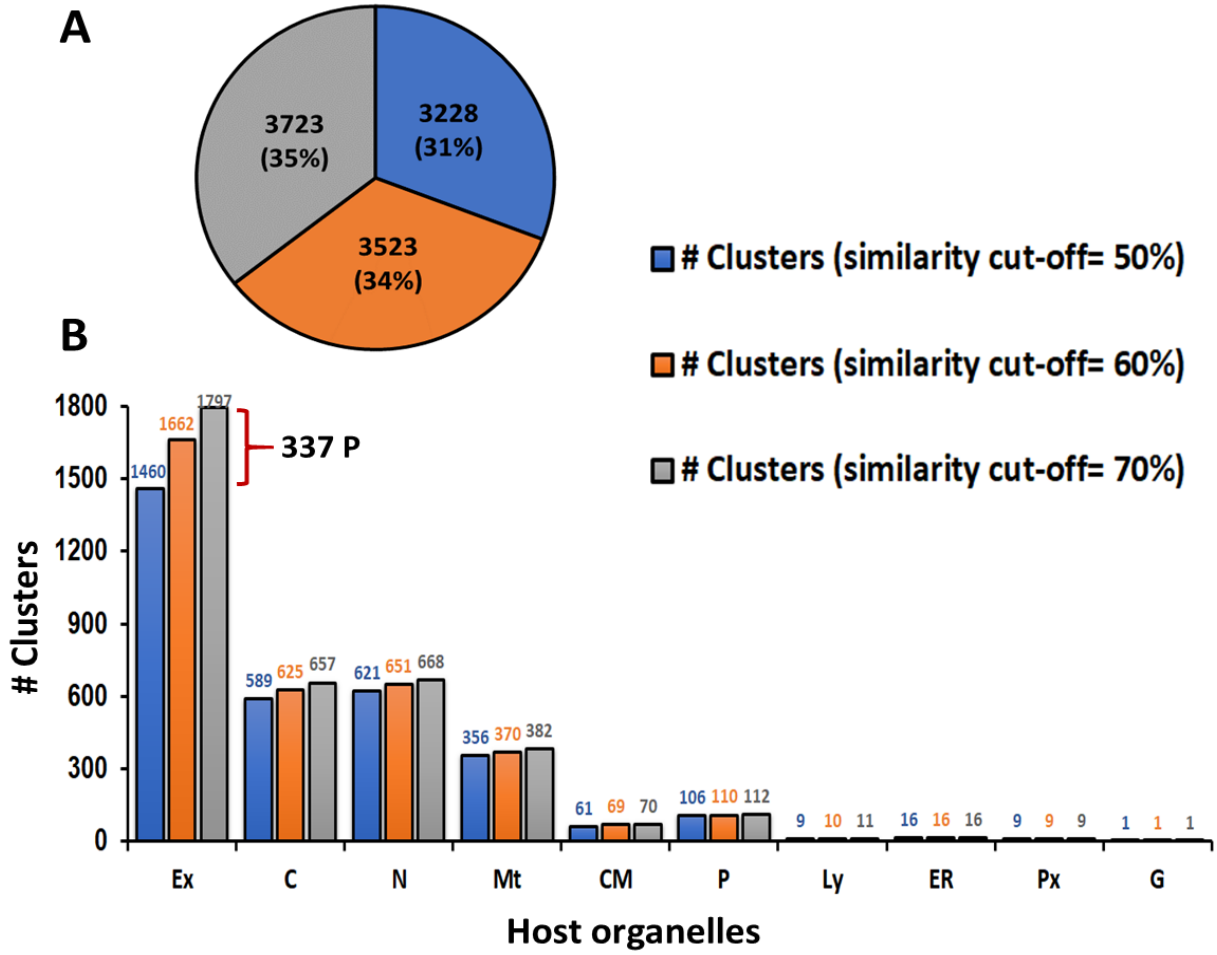

Fig. 2 – Clustering rust fungal effector protein sequences using CD-HIT. A. At three different sequence similarity cut-offs (50%, 60%, and 70%), the number of clusters ranged from 3228 at 50% to 3723 at 70%. B. Cluster analysis also revealed differences in the number of proteins targeted to various host cell subcellular localizations and organelles. The chosen 50% cut-off resulted in 1460, 589, 621, 356, 61, 106, 9, 16, 9, and 1 protein clusters predicted to localize in the extracellular space (Ex), cytoplasm (C), nucleus (N), mitochondria (MT), cell membrane (CM), plastid (P), lysosome (Ly), endoplasmic reticulum (ER), peroxisome (Px), and Golgi (G), respectively.

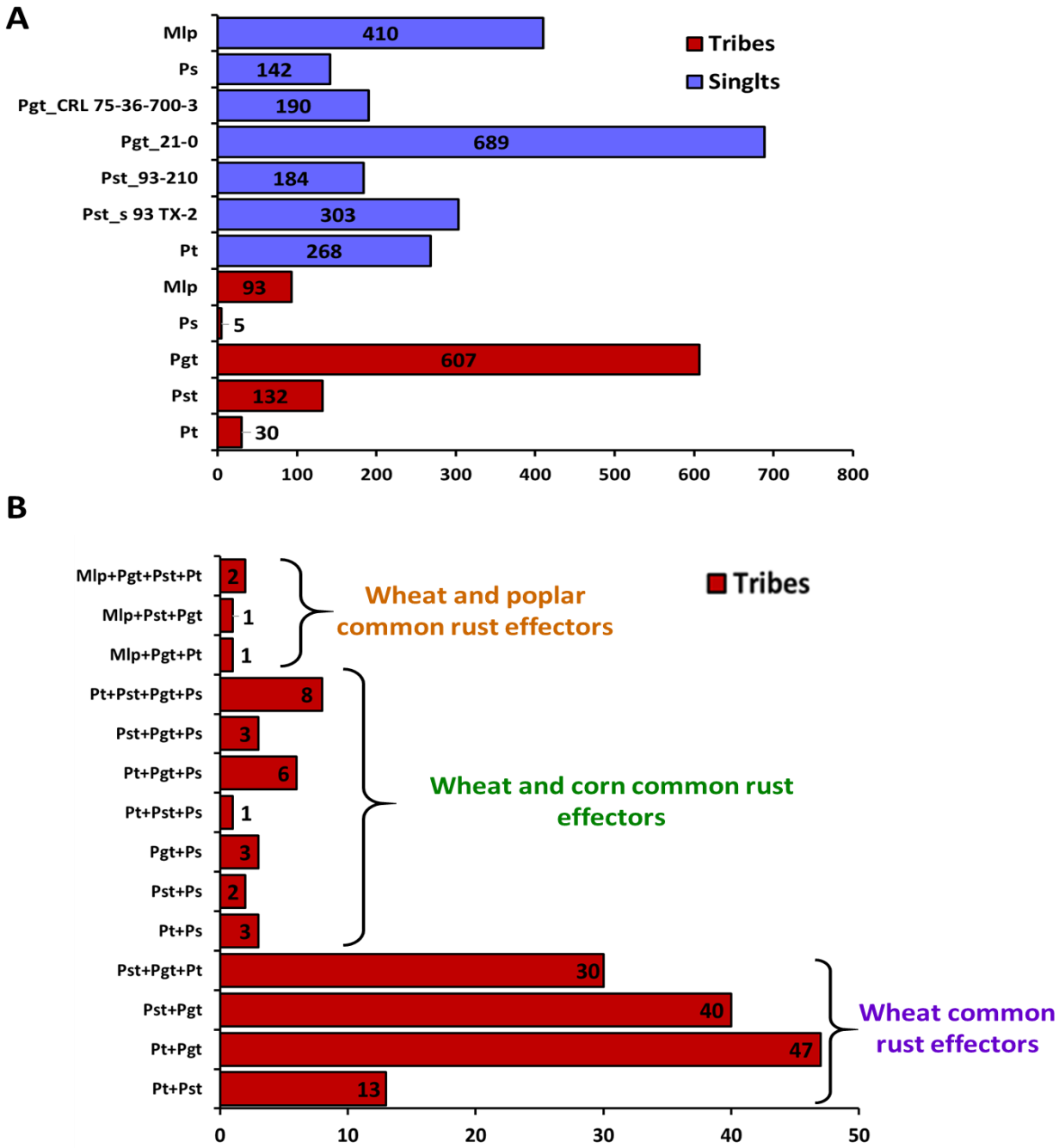

Fig. 3 – The distribution of rust effector singlets and tribes. (A) The numbers of singlets and species-specific tribes for investigated rust fungi. (B) Common tribes across the five rust fungi; wheat common rust effectors (Pt+Pgt+Pst, Pt+Pgt, Pt+Pst, and Pgt+Pst), wheat and corn common rust effectors (Pt+Pgt+Pst+Ps, Pgt+Pst+Ps, Pt+Pst+Ps, Pgt+Ps, +Pst+Ps, and +Pt+Ps), wheat and poplar common rust (Pt+Pgt+Pst+Mlp, Pgt+Pst+Mlp, Pt+Pgt+Mlp).

The effector proteins of both wheat and corn rusts are associated with the same genus, *Puccinia*. Upon studying the effector proteins of wheat and corn rusts, we identified eight tribes commonly shared among them (Fig. 3B). Among these eight tribes, two were found to have unknown functions but were predicted to localize in the nucleus. The other six *Puccinia*-specific tribes of effector proteins were predicted to be extracellular, with four having unknown functions and two having the functions of copper/zinc superoxide dismutase (C/ZSD) and thaumatin-like proteins (ThLPs). Furthermore, commonalities were observed between wheat and corn rusts, except for one or two wheat rust fungi. Among them, six tribes were commonly shared between Pt, Pgt,

and Ps, while Pst, Pgt, and Ps shared three tribes. One tribe was found to be common between Pt, Pst, and Ps.

The comparative analysis of all rust effectors revealed no common tribe between poplar and corn rusts. However, poplar rusts shared two tribes with all wheat rusts, namely Tribe_620 and Tribe_36 (Fig. 3B), that were predicted to localize to the cytoplasm and extracellular host cells and identified as peptidyl-prolyl cis-trans isomerase fpr2 (PPI-fpr2) and uncharacterized proteins, respectively. Moreover, similarities were identified between wheat and corn rusts, except for one wheat rust fungus. These include the presence of Mlp+Pt+Pgt in Tribe_624, predicted to localize in the cytoplasm, and Tribe_994, found in Mlp+Pt+Pst, predicted to be in the cell membrane.

Expression pattern of shared wheat rust effectors

We conducted an in-depth analysis of the gene expression profiles of the three candidate effector repertoires of the wheat rust fungi. The expression pattern of tribes common to the three wheat rust fungi was analyzed, with a specific focus on those tribes that are expressed across these fungi, aiming to understand their universal role in the infection processes. To achieve this, transcript mapping was utilized to quantify transcripts corresponding to these effector genes, using publicly available RNA-seq data obtained from the SRA. This data was specifically derived from wheat leaf samples infected with wheat rusts. The analysis focused on two key stages of leaf rust infection: 0 hours post-inoculation (hpi) and 24 hpi, allowing us to capture the temporal dynamics of gene expression during the early phases of pathogen invasion and colonization. After normalizing the transcript data, we compared the abundance of these candidate effector genes across different wheat genotypes. Through this comparison, we identified specific effector genes that may play a critical role in the rust infection process.

The expression profiling of the three-wheat rust fungal effector tribe repertoires provided valuable insights into the specific roles and dynamics of various effector tribes during the infection process. Among the 182 effectors identified across the three wheat rust fungi, only 62 were found to be expressed in at least one stage. Particularly noteworthy are effectors assigned to Tribe 1 and Tribe 7, which are induced by infection at 24 hpi (Fig. 4A). These tribes, which are likely localized in the apoplast, have not had their specific functions identified. Interestingly, these tribes were completely silent during the urediniospore stage, the resting spore stage of the fungus. Their activation post-infection suggests a possible role in the early stages of host colonization, where they may be involved in manipulating the host's immune responses or facilitating the establishment of infection. Other tribes displayed expression patterns that were restricted to one or two of the wheat rust fungi, indicating a level of specialization or divergence in effector function among the different rust species. This differential expression across species and stages highlights the complexity of the fungal infection process. In contrast to these infection-induced tribes, the Tribe_990 stood out for its consistent expression across all tested stages and in all three wheat rust fungi. Identified as a cupredoxin domain-containing protein localized in the cell membrane, Tribe 990's constitutive expression suggests it plays a fundamental role in the fungal life cycle, possibly involved in essential processes such as maintaining cell integrity.

The shared tribes between *P. triticina* and *P. graminis tritici* were also analyzed. Approximately 35% of the tribes showed expression at one stage of the infection process. Specifically, effectors assigned to Tribe 646, which localize to the cytoplasm, and Tribes 132 and 186, which are localized in the apoplast, all exhibited induced expression 24 hpi (Fig. 4B). In contrast, several other tribes were consistently expressed throughout the infection stages, including Tribe 993, Tribe 108, Tribe 924, Tribe 936, Tribe 949, and Tribe 1027. These tribes were predicted to localize to various cellular components, including the cell membrane (CM), extracellular space (EX), mitochondria (Mt), and plastids. Despite their consistent expression, the functions of these tribes remain largely unknown, with the exception of Tribe 1027, which is predicted to function as a copper/zinc superoxide dismutase, an enzyme involved in oxidative stress response in the plastid.

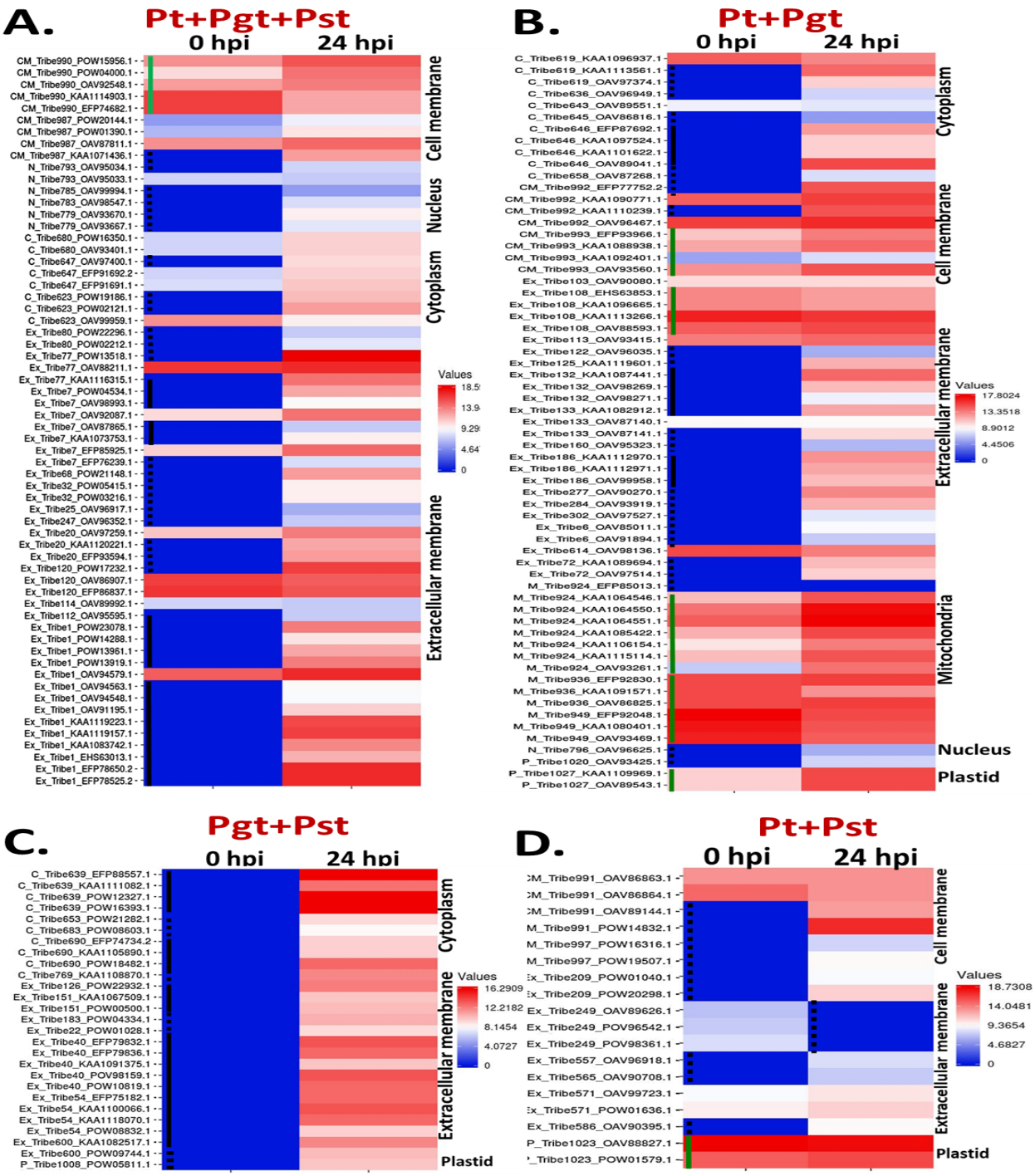

Fig. 4 – Expression profiling of effector tribes during wheat rust fungi infection. Comparison of effector gene expression assigned to shared tribes among rust fungi at 0 hours post-inoculation (hpi) and 24 hpi. A. Expression profile of effector genes belonging to tribes shared by Pt, Pgt, and Pst. B. Expression profile of effector genes in tribes shared by Pt and Pgt. C. Expression profile of effector genes in tribes shared by Pgt and Pst. D. Expression profile of effector genes in tribes shared by Pt and Pst.

The expression pattern of Pst displayed a distinct profile when compared to either Pgt or Pt. Unlike the consistent or early-stage expressions observed in Pgt and Pt, the shared tribes between Pgt and Pst were only induced after 24 hpi. These shared tribes include Tribe 639, Tribe 690, Tribe 600, Tribe 151, Tribe 40, and Tribe 54, all of which currently have unknown functions (Fig. 4C). On the other hand, effectors assigned to Tribe 1023, a shared tribe between Pt and Pst, exhibited

constitutive expression across the different stages of infection (Fig. 4D). Despite its consistent expression, the function of Tribe 1023 remains unknown, highlighting the need for further investigation to elucidate its role. This contrast in expression patterns between the tribes shared by Pgt and Pst and those shared by Pt and Pst underscores the complex and varied strategies employed by these rust fungi during infection and their adaptation to different stages of host interaction.

Discussion

Rust fungi employ various strategies to subvert host defenses during infection, among which the secretion of effector proteins stands out as an essential mechanism for orchestrating host-pathogen interactions. The availability of high-throughput 'omics' data has been a game-changer for the field, allowing for the identification and comparison of effectors across various rust species and strains. Predicting the secretome of fungi relies on the fundamental principles of identifying secretion signals and the absence of transmembrane helices. Here, we developed a comprehensive pipeline to predict candidate effector proteins for five rust fungi. This analysis yielded CSEPs for each rust fungus strain investigated. Notably, the percentage of the selected effector proteins ranged from 1% to 6%. The highest number of effector proteins was predicted in Pgt_210 (2286 proteins, 6%), while the lowest numbers were predicted in Ps_RO10H11247 (187 proteins, 1%). These findings were consistent with a study on *P. graminis* by Sperschneider et al. (2018), which revealed approximately 10% of the proteome to be predicted as effector proteins. These identified CSEPs could be a valuable resource for studying the interactions between rust fungi and their hosts and developing strategies to control rust diseases.

Deciphering the subcellular localization of fungal rust effectors is a critical factor in uncovering their mechanisms of interaction with host cells since it helps to determine the localization of the action of these effectors within the host cell. The utilization of DeepLoc revealed varied subcellular localizations for rust effectors. Recent studies have underscored the complex interplay between fungal rust effectors and host cells, including their tendency to target various cellular compartments (Gladieux et al. 2014, Lorrain et al. 2019, Wang et al. 2020). Our findings contribute to this growing understanding by providing a holistic perspective on rust effector localization diversity. The classification of fungal rust effectors provides comprehensive insights into their evolutionary dynamics and functional implications in host-pathogen interactions. Employing clustering algorithms, notably CD-HIT, to analyze diverse rust effector protein sequences at a similarity cut-off of 50% revealed consistent clustering patterns across cut-offs, indicating the robust capture of evolutionary relationships. The resulting cluster numbers ranged from 3228 to 3723 clusters and aligned with previous research favoring lower cut-offs for identifying orthologs across diverse species (Singh et al. 2021). This choice leads to the prediction of protein clusters localized in different compartments. The clustering generates 2186 singlets and 1027 tribes, displaying diverse molecular weights and sizes. This diversity in effector protein structure was observed in other plant-pathogen interactions and was thought to contribute to the ability of pathogens to overcome host defense (Dou & Zhou, 2012, Bhurta et al. 2022). Our dataset possesses diverse structural and physical characteristics in the context of host-pathogen interactions. Rapid evolution is a common feature of rust fungal effectors and has been attributed to various factors, including their high rates of mutation and recombination as well as the selection pressure imposed by host resistance mechanisms (Franco et al. 2022). The high degree of singlet effector divergence observed in some rust effectoromes reflects the ongoing arms race between the pathogen and its host, emphasizing the importance of effector diversity and rapid evolution in the success of rust pathogens as plant pathogens. Moreover, shared tribes could offer insights into pathogen adaptation and evolution, as a recent study revealed that species-specific effectors play a crucial role in the pathogenesis and virulence of rust fungi, with some effectors even being host-specific (Jan et al. 2021). These common tribes may provide a foundation for understanding the molecular mechanisms underlying pathogen adaptation and evolution.

The conservation of common wheat rust effector proteins holds significant implications for devising effective strategies against the prevalent wheat rust disease. Comparative analysis across

different wheat rust species revealed the presence of conserved tribes shared among them. An approximately 30 conserved tribes were found across the three *Puccinia* species that specifically infect wheat. These conserved effectors could represent essential components of the pathogenicity machinery of wheat rust fungi and are likely to play important roles in manipulating host plant defense and facilitating infection. Surprisingly, 18 of these conserved tribes are predicted to function extracellularly, emphasizing the need for deeper investigation into their roles in the rust-wheat interaction. In addition, six tribes were found in the cytoplasm, four in the nucleus, and two in the cell membrane. Among these, TLPs and CMs stand out. TLPs in fungi are thought to play a role in manipulating host cells to create an environment favorable for infection (Üstün et al. 2016). In their investigation, TLPs were found to be involved in redox signaling and regulation, which could affect gene expression and cellular processes in the host plant cells. In wheat, TLPs were identified in several species of wheat rust fungi, including *P. striiformis* and *P. triticina* (Ortiz et al. 2022, Xia et al. 2022). The studies suggested that these proteins could play a role in the pathogenicity of the fungi by regulating the redox environment within the host plant cells. Similarly, CMs are involved in the biosynthesis of several defense-related compounds, including salicylic acid, which could play a critical role in plant defense against pests (Liu et al. 2022). Both TLPs and CMs could be potential targets for genetic engineering to enhance plant resistance against wheat rust fungal infections. By manipulating the expression levels of these genes, it may be possible to enhance the plant's natural defense mechanisms and improve their ability to resist infection. In addition, the targeting of PSMB2 to the nucleus is believed to play an important role in bacterial infection. This protein was shown to be upregulated during infection and was found to interact with other proteins involved in the regulation of gene expression (Cui et al. 2021). It was thought that PSMB2 may play a role in the degradation of host defense proteins, allowing the pathogen to establish infection and evade the plant's immune response (Cui et al. 2021). Further research on this protein and its interactions during wheat rust fungal infection could also lead to the development of new strategies for controlling this devastating disease. Similarities in shared tribes between different rust species suggest varying degrees of evolutionary relatedness, with Pgt and Pt exhibiting a closer relationship compared to other pairs (Zhang et al. 2018). These findings underscore the importance of conserved effectors in pathogenicity and highlight potential targets for devising innovative approaches to combat wheat rust.

The presence of shared tribes among effector proteins from wheat and corn rusts within the same *Puccinia* underscores the intriguing interplay between these fungal pathogens and their respective host plants. In our analysis, eight conserved tribes were identified, shedding light on potential mechanisms of infection and host manipulation. Notably, nuclear-targeting effectors within these tribes could play pivotal roles in gene expression regulation, mirroring the function of known wheat rust effectors like AvrSr50. AvrSr50 functions as a transcriptional activator that targets the host plant nucleus and induced the expression of genes involved in susceptibility to rust infection (Zhang et al. 2018). Understanding the mechanisms by which nuclear-targeting effectors function and identifying their specific host targets could lead to the development of novel strategies for controlling their actions in the nucleus.

Additionally, six *Puccinia*-specific tribes were predicted to function extracellularly, with functions including C/ZSD and ThLPs. Recent studies revealed that some fungal pathogens, including rust fungi, can also secrete thaumatin-like proteins to suppress the host plant's immune response (Shaw 2002). By binding to the plant's surface receptors, these effector proteins could interfere with the recognition of fungal cell wall components and inhibit the activation of defense signaling pathways (Kamel et al. 2023). This interference may allow the rust fungus to evade the host plant's immune system and establish a successful infection. These extracellular effectors may contribute to the suppression of host immunity and the promotion of fungal growth by interfering with host signaling pathways. Moreover, shared tribes among Pt, Pgt, and Ps, as well as among Pst, Pgt, and Ps, emphasize the commonalities and evolutionary relationships among these rust species. Overall, the discovery of shared tribes among wheat and corn rust effectors in the genus *Puccinia* underscores the need for continued research into the mechanisms of pathogenesis and host

adaptation in rust fungi, with the ultimate goal of developing effective and sustainable strategies for rust disease control in crops.

In contrast, the comparison between poplar and wheat rusts revealed divergence, with only two shared tribes between them. The identification of these shared tribes, particularly PPI-fpr2, opens avenues for exploring mechanisms of immunophilin-based manipulation of the host plant's immune system. The function of PPI-fpr2 in rust fungi is not yet fully understood, but it has been hypothesized to play a role in manipulating the host plant's immune system. A recent study demonstrated that PPI-fpr2 interacted with the extracellular matrix to promote the folding of peptides and proteins (Lin et al. 2019, Khalil et al. 2024). By altering the conformation of these proteins, this effector protein may be able to interfere with their proper functioning and inhibit the activation of defense responses. This may allow the rust fungus to establish a successful infection and colonize the host plant. Our findings suggest that the effector proteins of wheat and poplar rusts have been relatively divergent, reflecting the differences in their evolutionary histories and host specificity. Moreover, the functional identification and localization of the shared tribes provide insights into the potential roles of these effectors in the common pathogenesis and host adaptation of both rust fungi species.

The analysis of gene expression profiles among the three wheat rust fungi has unveiled critical insights into their infection strategies and effector functions. With 182 shared effector tribes identified, only 62 were expressed during infection, emphasizing a selective activation of genes that likely play pivotal roles in manipulating host defenses. Notably, Tribe 1 and Tribe 7 were induced at 24 hours post-inoculation, suggesting their involvement in early colonization efforts, while Tribe 990 exhibited constitutive expression across all stages, indicating its fundamental role in the fungal life cycle. The observed specialization among tribes, where some were exclusively expressed in certain rust species, highlights the evolutionary adaptations of these pathogens to their respective hosts. This aligns with findings from a recent study that analyzed gene expression changes in different wheat varieties, which identified upregulated genes associated with stress responses and signaling pathways crucial for rust resistance (Lee et al. 2022, Nazarov et al. 2024). Furthermore, the identification of constitutive effectors like Tribe 990, which maintains expression across all stages, indicates fundamental roles in the fungal life cycle, paralleling insights from the rust expression browser project that has compiled extensive RNA-seq data to elucidate host responses to infection (Adams et al. 2021). Collectively, these studies underscore the complexity of fungal infection strategies and highlight the need for further research to elucidate the specific roles of these effectors in enhancing wheat resistance against rust diseases. This complex interplay between effector expression and host interaction underscores the necessity for further research to elucidate the specific roles of these effectors, which could significantly inform breeding strategies aimed at enhancing wheat resistance to rust diseases.

Despite the fact that few proteins have been identified with specific functions, the majority of these proteins with unknown functions are believed to be critical players in wheat pathogenicity. The identification of these unknown tribes presents an exciting opportunity for further research, which may aid in developing effective strategies for coping with these pathogens and potentially serve as a component of resistance. Understanding the functions of these proteins could reveal new insights into the mechanisms of wheat-rust interactions, providing a foundation for the development of novel and effective control methods. Additionally, the conservation of these effectors across different rust fungal species may underscore the importance of these proteins in the pathogenesis of rust diseases, suggesting their potential as targets for the development of broad-spectrum control measures. In addition, CRISPR technology provides a powerful tool for modifying the wheat genome and altering the function of these effectors. With the use of CRISPR, we can target specific genes to introduce mutations that prevent these effector functions. This could potentially lead to the development of wheat plants that are resistant to rust infections. Therefore, the investigation of the functional analysis of these common wheat rust effectors may hold significant implications for the future of crop protection and agricultural sustainability. Future research could also explore the incorporation of other data types, such as transcriptomic and

proteome data. These data can provide insight into which effectors are actively expressed during infection and help identify novel functions.

Conclusions

Effector proteins secreted by wheat rust fungal pathogens during plant infection are believed to be important in host-pathogen interactions. The use of high-throughput omics data has revolutionized the field of effector biology by allowing for the identification and comparison of effectors across different rust species. In this study, we analyzed total protein datasets of three wheat rust fungi to identify candidate secreted effector proteins and expanded the analysis to include corn and poplar rusts to gain a broader understanding of rust pathosystems. The findings showed a diverse range of subcellular localizations for rust effectors, but many predicted to target extracellular locations. Our study revealed a high degree of singlet effector divergence in all effectoromes, reflecting the ongoing arms race between the pathogen and its host. The identification of conserved effectors shared between rust fungi provides a valuable resource for developing effective control strategies against rust diseases. Of the 30 common tribes of effector proteins conserved across the three wheat rust fungi, 18 were predicted to have functions outside of wheat cells, highlighting the need for further investigation into their specific roles in the *Puccinia*-wheat interaction. Some of the identified effector proteins, including TLPs, CMs, and PSMB2, may be potential targets for genetic engineering to enhance plant resistance against wheat rust fungal infections. The study of *Puccinia*-specific effectors in rust fungi has also revealed the presence of shared tribes among wheat and corn rusts, suggesting a common mechanism of pathogenesis and host adaptation to rust fungi. Of eight common tribes, two were identified to have the functions of C/ZSD and ThLP, which have been shown to protect fungal cells from the host plant's immune response to establish a successful infection. While there were no common tribes between poplar and corn rusts, two tribes were found to be shared between poplar and all wheat rusts, which were predicted to localize to the cytoplasm and extracellular host cells and identified as PPI-fpr2 and uncharacterized proteins. PPI-fpr2 has been shown to interact with plant proteins involved in defense signaling pathways and may play a role in manipulating the host plant's immune system to promote fungal infection. Utilizing publicly available RNA-seq data from wheat leaf samples infected with these pathogens, we analyzed the expression patterns of 182 shared effector tribes across *Puccinia triticina*, *Puccinia graminis* f. sp. *tritici*, and *Puccinia striiformis* f. sp. *tritici*. Our analysis centered on two critical stages of leaf rust infection: 0 hours post-inoculation (hpi) and 24 hpi, allowing us to capture the temporal dynamics of gene expression during the early phases of pathogen invasion and colonization. Among the identified tribes, only 62 were expressed at least at one stage, with Tribe 1 and Tribe 7 showing significant induction at 24 hpi, suggesting their involvement in early host colonization. Interestingly, these tribes were silent during the urediniospore stage, indicating a strategic activation that may facilitate infection by manipulating host immune responses. It is important to understand the role of effectors in the evolution of plant-pathogen interactions. This could involve exploring the coevolution of effectors and their corresponding host resistance genes, as well as investigating the role of horizontal gene transfer in effector evolution.

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