



Intrinsically Disordered Kiwellin Protein-Like Effectors Target Plant Chloroplasts and are Extensively Present in Rust Fungi

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Abstract

The effector proteins produced by plant pathogens are one of the essential components of host–pathogen interaction. Despite being important, most of the effector proteins remain unexplored due to the diversity in their primary sequence generated by the high selection pressure of the host immune system. However to maintain the primary function in the infection process, these effectors may tend to maintain their native protein fold to perform the corresponding biological function. In the present study, unannotated candidate secretory effector proteins of sixteen major plant fungal pathogens were analyzed to find the conserved known protein folds using homology, ab initio, and Alpha Fold/Rosetta Fold protein dimensional (3D) structure approaches. Several unannotated candidate effector proteins were found to match various known conserved protein families potentially involved in host defense manipulation in different plant pathogens. Surprisingly a large number of plant Kiwellin proteins fold like secretory proteins (> 100) were found in studied rust fungal pathogens. Many of them were predicted as potential effector proteins. Furthermore, template independent modelling using Alpha Fold/Rosetta Fold analysis and structural comparison of these candidates also predicted them to match with plant Kiwellin proteins. We also found plant Kiwellin matching proteins outside rusts including several non-pathogenic fungi suggesting the broad function of these proteins. One of the highest confidently modeled Kiwellin matching candidates effectors, Pstr_13960 (97.8%), from the Indian *P. striiformis* race Yr9 was characterized using overexpression, localization, and deletion studies in *Nicotiana benthamiana*. The Pstr_13960 suppressed the BAX-induced cell death and localized in the chloroplast. Furthermore, the expression of the Kiwellin matching region (*Pst_13960_kiwi*) alone suppressed the BAX-induced cell death in *N. benthamiana* despite the change of location to the cytoplasm and nucleus, suggesting the novel function of the Kiwellin core fold in rust fungi. Molecular docking showed that Pstr_13960 can interact with plant Chorismate mutases (CMs) using three loops conserved in plant and rust Kiwellins. Further analysis of Pstr_13960 showed to contain Intrinsically disordered regions (IDRs) in place of the N-terminal $\beta 1/\beta 2$ region found in plant Kiwellins suggesting the evolution of rust Kiwellins-like effectors (KLEs). Overall, this study reports the presence of a Kiwellin protein-like fold containing a novel effector protein family in rust fungi depicting a classical example of the evolution of effectors at the structure level as Kiwellin effectors show very low significant similarity to plant Kiwellin at the sequence level.

Keywords Rust effectors · Secretome · Structural genomics · Kiwellin proteins · Cell death

Introduction

All plant fungal pathogens use secretomes, and their independent entities, commonly known as effectors as an essential weapon to defeat the host resistance mechanism irrespective of nutritional style. It is now evident through various studies that understanding the secretome can provide the solution to reduce losses caused by plant pathogens [1–3]. Moreover, with the beginning of genome sequencing studies, unraveling the pathogen secretome has become very

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much feasible. A large number of economically important necrotrophs, hemibiotrophs, and biotrophs have been studied to elucidate the secretomic differences and further potential avirulence candidates to develop resistant varieties [1, 4]. However, irrespective of large genomic resources and various computational tools, the complete exploration of the pathogen secretome is accompanied by several hurdles. The major problem in understanding the secretome is the absence of conserved features among the effector proteins, especially for obligate biotrophic pathogens [1, 5]. During host–pathogen interaction, the host immunity genes impose selection pressure on the effector encoding genes leading to changes in their gene sequences for avoiding the recognition of their protein product continuously by the host resistance proteins [6, 7]. Due to continuous selection pressure imposed by the host, these genes evolve extensively and diverge from their original family members causing their identification challenging using sequences-based search approaches. Though, selection pressure changes the effector sequence over time; to continue their native functions, these effectors may tend to keep their conserved functional fold essential for their original function rather than maintaining the full domain [8]. Intriguingly few studies have recently revealed the molecular function of the effectors could be predicted due to their conserved 3 Dimensional fold matching to known effector family despite no sequence similarity at the sequence level [8].

Recently, the structural genomics of two poplar rust effectors, *MLP124266* and *MLP124017*, using nuclear magnetic resonance (NMR), showed similarity to knottin and nuclear transport factor 2-like proteins [9]. Similarly, the MAX effector family includes *AVR1-CO39* (*Magnaporthe oryzae*), *AvrPiz-t* (*M. oryzae*), and *ToxB* effectors (*Pyrenophora tritici-repentis*) [8], and several other effectors present in many ascomycetes pathogens that do not share similarity among themselves at the sequence level, however possess a conserved six β -sandwich fold, stabilized by a disulfide bridge between 2 conserved cysteines residues [10, 11]. Similarly, several other effectors from *Blumeria graminis* are known to exhibit similarity to ribonucleases (RNase-like effectors) and are involved in host defense manipulation [12]. These studies have clearly shown that the arms race has led pathogens to diverge the primary sequence of effectors; simultaneously, functional necessity has sustained these proteins to keep conserved folds [8, 12].

To identify such unknown conserved folds containing novel effectors secretome-wide structural modeling of effectors has been proved to be very useful recently [13]. Structural genomics of rice blast pathogen *Magnaporthe oryzae* secretome using template-based and template-free modeling predicted the 70% secretome having known protein folds associated with various biological functions [14]. Moreover, the discovery of the Alpha Fold and RoseTTA FOLD has also introduced template-free modeling that can generate

a highly confident 3D structure without prior knowledge [15, 16]. Alphafold, mediated structural genomics of two important plant pathogens *Fusarium oxysporium* and *Venturia inaequalis* discovered a large number of folds in the candidate effectors that match the previously identified effector families such as MAX, ToxA, LARS, and FOLD further emphasizing the role of structural genomics in highlighting effector functions [17, 18]. A comparative Alphafold-driven structural genomics of plant pathogens and thereafter structure-based clustering cataloged several orphan effector candidates to known effector families such as hydrophobins, RxLR, RNase, ToxA, and several others. Over all, comparative structural genomics therefore can highlight effectors that are difficult to identify by the homology-based search using the primary sequence of these candidates [19].

Kiwellins proteins are members of a cysteine-rich major allergen protein family, present in kiwi fruits and reported to cause allergic reactions in humans after consumption [20]. In addition to their role as an allergen in kiwi plants, no designated role was assigned to those proteins until Han et al., (2019), proposed their role in the host defense against plant pathogens [21–23]. Kiwellins homologous are also reported in all major plant species, including conifers, and angiosperms. These proteins are reported to be present in high members suggesting the expansion of this family [21]. The study by Han et al., (2019) defined these proteins to block the functioning of the fungal effector, *Cmu1*, known to down-regulate the salicylic acid synthesis by channeling its substrate to other pathways [21–24]. Han et al. (2019) showed that *ZmKWL1*, one of the Kiwellin from the 20 encoded Kiwellins by the *Zea mays* (maize), binds to the *Cmu1* active site so that *Cmu1* could not bind to its substrate or dimerize with host Chorismate mutase (CMs). The study also defined that the plant Kiwellin possesses unique folds that specifically bind to fungal *Cmu1* rather than their native CMs.

To our knowledge, no candidate secretory effector proteins (CSEPs) have been reported to possess Kiwellin-like folds in the effector proteins. In the present study using comparative structural genomics of plant pathogens belonging to different lifestyles, we report Kiwellin protein-like fold-containing effectors (KLEs) in different rust fungi. We showed that rust and other plant pathogens may possess Kiwellin proteins like fold. The structural comparison also showed that plant Kiwellin and rust KLEs possess a central carbohydrate binding core similar to previous studies however rust KLEs are more similar overall 3D fold to plant Kiwellin than Barwin-like proteins/endonucleases. The analysis of the rust KLEs also revealed them to possess N-terminal intrinsic disordered regions (IDRs) of variable lengths where the $\beta 1/\beta 2$ region is present in plant Kiwellins. Further, we also showed that *Pstr_13960* one of the rust KLEs from *Puccinia striiformis* race YR9 can suppress BAX-induced cell death in

Nicotiana benthamiana. The deletion fragments analysis revealed N-terminal IDR containing region is involved in the chloroplastic targeting of *Pstr_13960*. The Kiwellin core matching region of *Pstr_13960* alone also independently suppressed the BAX-induced cell death in *N. benthamiana* despite its changed location in the cytoplasm and nucleus. Further, the docking analysis of host chorismate proteins (CMs) with rust Kiwellin effectors showed the positive interaction of these proteins, suggesting that the rust effector may capture the host CMs as an alternate strategy to block salicylic acid synthesis. The present work is the first study to report the presence and evolution of Kiwellin-like-fold effectors in rust fungi and their expansion in their secretomes. The study further highlights the use of the KLEs to target chloroplast and exhibits cell death suppression possibly binding by interacting with plant chorismate mutase protein.

Materials and Methods

Secretome Analysis and Identification of Novel CSEPs in the Proteome of Plant Pathogens

The predicted proteome data of plant fungal pathogens was retrieved from Ensembl fungi, except for *Puccinia triticina*, and *P. striiformis*, which were utilized from Kiran et al. [25, 26]. The genome of *P. arachidis* and *P. horiana* was downloaded from the NCBI database, and genes were predicted using MolQuest 2.0 (FGENESH) (PRJNA280565, PRJNA306202). The sequence of *Zea mays* and *N. benthamiana* different Kiwellins were retrieved from the UniProt database. The secretome analysis was carried out using the secretool online server with default parameters [27]. The conserved domain analysis was done using the Pfam and NCBI-CD Batch search facility using default parameters [28, 29]. The candidate proteins with no annotations were treated as novel secretory proteins. These proteins were further analyzed using EffectorP 2.0 software for potential effector candidates [30]. The proteins with scores above 0.5 were treated as potential candidate effectors.

Structure Prediction, Comparative Analysis, Docking of CSEPs

The template-based modeling of the CSEPs was done using the PHYRE2 protein fold recognition server, Robetta, whereas ab initio modeling was carried out using I-TASSER [31–33]. The 3D structure with template similarity of a minimum of 20% and the confidence score of > 60% proteins were counted as significant fold similarity in PHYRE2 analysis. The hits with less than the threshold score were removed from the further analysis. The Ramachandran plot and model stability were checked using the SAVES server (<https://saves.mbi.ucla.edu/>)

To further analyze the results obtained by PHYRE2, Robetta, and I-TASSER all the Kiwellin folds containing candidate secretory proteins were analyzed using the Alpha Fold and RoseTTA Fold structure prediction method using the Colab server and online server [16, 34]. The pTM score > 0.5 and pLDDT score > 70 were considered as the threshold for the correct AlphaFold structure prediction. The fold conservation analysis of candidate structures obtained after PHYRE2 and alpha fold analysis was done using mTMalign, Foldseek, and YASARRA softwares. The docking analysis was performed using default parameters using FRODOCK [35].

Identification, Sequence Alignment, and Motif Analysis of Kiwellin Protein Fold Containing Candidate Rust Effectors

The sequence alignment of full-length proteins and the conserved region was done using MAFFT software [36]. Motif analysis was done using the MEME motif at default settings (<https://meme-suite.org/meme/doc/cite.html>). [37] For comparative analysis, the plant Kiwellin proteins were downloaded from NCBI. To identify Kiwellin-like proteins in other plant pathogens and other rust races, we performed genome-wide identification using randomly selected rust Kiwellin and plant Kiwellin proteins against the NCBI nr database, MycoCosm fungi genome database. The significantly matched proteins (e-5) were selected for structural, secretory, and effector prediction analysis.

Phylogenetic Analysis, Gene Structure Cluster, Network, and Expression Analysis.

The intron–exon boundaries were predicted using the GSDS server (<http://gsds.gao-lab.org/>). The MAFFT software was used for aligning the rust fungi-specific Kiwellin-like effector proteins, and an aligned file was used to generate a maximum likelihood method-based phylogenetic tree using the IQ-TREE server (<http://iqtree.cibiv.univie.ac.at/>).

Disordered Region and Transient Peptide Prediction Analysis

The disordered region analysis was done using, SLIDER, PONDER, and IUpred2A tools using default parameters [38–40]. The disordered region of candidate effectors was further confirmed by unfolded region after Alphafold analysis. The secondary structure analysis of the candidate structure was done using PDBSum. The transient peptides were predicted using ChloroP, Localizer, and WolfPsrt [41–43].

Cloning of Candidate Rust KLE Genes and Fragment Deletion Analysis

All primers used in the study for plasmid construction are shown in Supplementary Table 1. The RNA was extracted from *Puccinia striiformis* YR9 infected wheat leaf tissue samples using mirvana RNA isolation kit (USA). The coding sequence (CDS) of *Pstr_13960* (with and without signal peptide coding sequence) was PCR amplified using gene-specific primers using the cDNA synthesized from the aforementioned RNA samples (Primers and PCR conditions information Supplementary Table 1). The PCR amplified sequences were cloned in the pENTR/D-Topo vector followed by confirmation with restriction and sequencing. The confirmed *pENTR_13960* plasmid clone was further mobilized in pGWB408 (overexpression) and pGWB441 (subcellular localization) using LR clonase enzyme mix (Invitrogen, USA). The Kiwellin domain matching region and various deletion fragments were also cloned for overexpression and localization studies similarly as mentioned for full length *Pstr_13960* (Primers sequence and PCR conditions information Supplementary Table 1). Full length CDS of the *BAX* gene was PCR amplified from mouse cDNA and further cloned in the pGWB408 vector.

Agroinfiltration, Subcellular Localization Overexpression, and Phenotype Analysis

Escherichia coli Topo10 cells (Invitrogen, USA) were used for performing all the cloning experiments. *N. benthamiana* plants grown at 22 °C with a 14 h light/10 h dark cycle and 3–4 weeks old plants were used for performing agroinfiltration assays. The *Agrobacterium tumefaciens* strain GV3101 chemically competent cells were transformed with *Pstr_13960*_pGWB441 (subcellular localization) and *Pstr_13960*_pGWB408 (overexpression) and other deletion constructs. The resulting transformants were further confirmed by PCR. The confirmed agrobacterium localization and overexpression construct clones were overnight grown in LB media (28 °C) and adjusted to an optical density (OD) of 0.4, centrifuged for 10 min at 4000 × g. The pellet was re-suspended in MES-K buffer (100 μM acetosyringone, 10 mM MgCl₂, and 10 mM MES, pH 5.6) and further incubated for five hrs at room temperature before infiltration on *N. benthamiana* leaves. For cell death suppression analysis, the candidate genes were infiltrated into *N. benthamiana* leaves 24 h later, and BAX infiltration was done at the same spots on the leaves. The phenotype was observed after 3–6 post infiltration.

Results

Structural Modeling of Orphan Unannotated Effector Candidates Highlight Similarities to Known Effector Family Members

The pipeline used for identifying novel secretory effector proteins and further structural modeling for identifying a candidate with the novel protein folds is shown in Fig. 1 (material and method section). The structural analysis of the unannotated secretome (Supplementary Table 2). showed that several candidates possess a similar fold as known effector family members. Among these, the RNA binding effectors, Microbial ribonucleases, Lytic polysaccharide monooxygenase, Gamma-Crystallin-like, Kiwellin fold proteins, knottins, RxLR, and Thaumatin-like were on the top list (Table 1). The number of candidates varied among the studied species where the highest no. of candidates were found for the Lytic polysaccharide monooxygenase (LPMO) family having 11 members in *P. sojae* (Table 1). Following *P. sojae*,

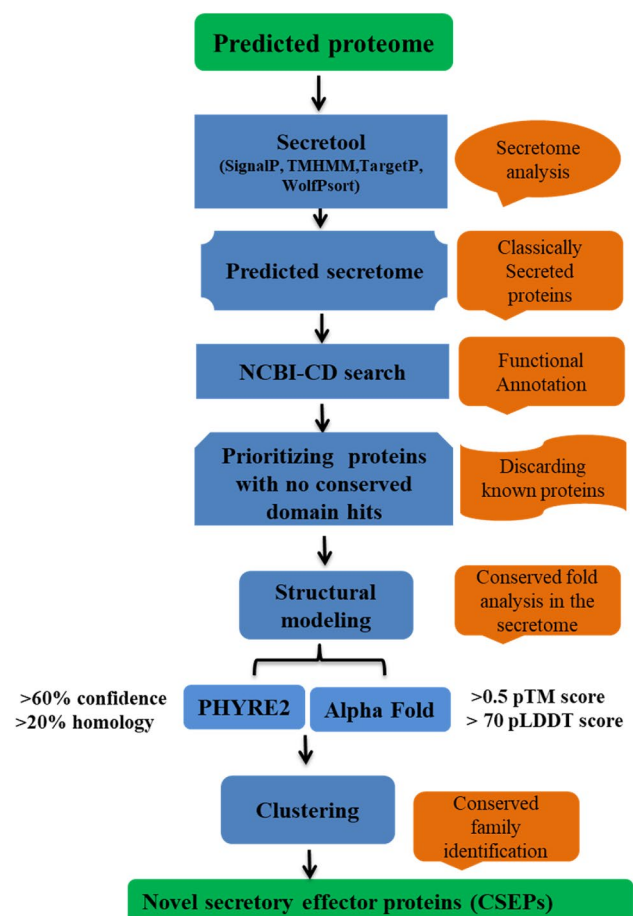


Fig. 1 Secretome and annotation pipeline for identification of novel candidate secretory effector proteins (CSEPs) in various studied species

Table 1 Top 10 conserved folds found in unannotated candidates secretory proteins of various studied plant pathogens

S.no	Protein Fold Name	Putative function	Organism name	Predicted secretory/effector proteins
1	RNA binding protein	Nucleic acid-binding	<i>B. graminis</i>	8
2	Microbial ribonucleases	RNA metabolism	<i>B. graminis</i> and <i>R. solani</i>	7,1
3	Lytic polysaccharide monooxygenase	Oxidative degradation of biopolymers such as chitin	<i>P. infestans</i> , <i>P. sojae</i>	5,11
4	Gamma-Crystallin-like	Diverse biological functions	<i>P. sojae</i> , <i>P. infestans</i> and <i>B.cinerea</i>	5, 2,1
5	Kiwellin like	Plant defense	<i>P. trititina</i> , <i>P. graminis</i> , <i>P. striiformis</i>	6,5,9
6	Avh146	Host defense manipulation	<i>P. infestans</i>	5
7	Avr3a4	Host defense manipulation	<i>P. infestans</i>	4
8	Scorpion toxin-like (knottins)	Diverse biological activity such as inhibitors, anti-microbial peptides, toxins	<i>P.sojae</i> and <i>M.oryzae</i>	3, 1
9	Thaumatin-like	Defense and developmental process	<i>R. solani</i> , <i>B. graminis</i> , <i>M. oryzae</i> and <i>B.cinerea</i>	2,2,1, 1
10	Smp-1-like	Cysteine protease calpain associated fold	<i>P. coronata</i>	2

Table 2 Secretory and effector analysis of Kiwellin matching effector proteins of rust fungi

Organism Name	Proteins matching with Kiwellin proteins like fold	Secretory extracellular predicted proteins	Proteins with effector-like features (Effector P 2.0)
<i>Puccinia graminis</i> UG99 A	12	8	8
<i>Puccinia graminis</i> 40–3	12	5	3
<i>Puccinia trititina</i> 77–5	11	6	5
<i>Puccinia trititina</i> Race1	16	11	10
<i>Puccinia striiformis</i> Yr9	14	9	8
<i>Puccinia striiformis</i> 104 E137 A	35	28	26
<i>Puccinia coronata</i>	21	18	17
<i>Melampsora larici-populina</i>	15	11	8
<i>Melampsora allii-populina</i>	10	7	6
<i>Puccinia horiana</i>	7	3	2
<i>Puccinia sorghi</i>	9	3	3
<i>Puccinia psidii</i>	3	1	1
<i>Phakopsora pachyrhizi</i>	1	1	1
<i>Hemileia vastatrix</i>	1	1	0

we found 5–9 candidates matching Kiwellin fold proteins in the case of *Puccinia* species (*P. trititina*, *P. graminis*, *P. striiformis*) that have been previously reported to present in defense-related genes in plants however no such finding has been reported for effectors candidates.

A Variable Number of Kiwellin-Like Effector Proteins are Present in the Different Rust Species

Among all the studied species, rust fungi possess candidate effectors that were matching plant defense-related

proteins We found several candidates matching Kiwellin proteins that are previously in eukaryotes, such as plants (Supplementary file S1) (modeling parameter, > 20% similarity, > 60%, modeling confidence). The initial Kiwellin effector candidates identified by PHYRE for *P. trititina*, (6) *P. graminis* (5) and *P. striiformis* (9). were also confirmed by I-TASSER and Robetta for further strengthening the notion of the similarity of these candidates to plant Kiwellin proteins. In light of these results, to further establish the Kiwellin effector's presence in all the major rust pathogens, these proteins were mined across all other

publically available rust genomes (Table 2). The analysis returned a variable number of proteins similar to Kiwellin proteins (minimum 1 and maximum 35 per rust species) (Table 2). Further, the NCBI CD- search revealed few of these proteins to possess DPBB (double psi beta-barrel) at the sequence level (Supplementary file S2).

Further, the structural modeling analysis revealed that most of these proteins match with plant Kiwellin protein fold (Supplementary file S3). As the DPBB fold is present in several other proteins such as endolytic peptidoglycan transglycosylase RlpA, EG45-like domain proteins, Barwin-like proteins, plant Kiwellins and expansins, bacterial papain inhibitors, and endonucleases. We designated only those proteins with Kiwellin-containing folds, where the top 3 hits of modeled protein matched with the crystal structure of plant Kiwellins and ripening-related proteins. The rest of the non-specific hits were discarded. Further, secretory and effector analysis of significantly matching candidate Kiwellin-like proteins revealed most of these were predicted to be secretory (1 to a maximum of 28 proteins per rust species genome) and effectors (1 and maximum of 26 proteins in rust species genome) (Table 2). The structural analysis revealed these proteins to have variable similarity and modeling confidence when compared to plant Kiwellins. The highest sequence similarity (45%) was demonstrated by PLW22586.1 and PLW05486.1 (*P. coronata*) and modeling confidence (97.8%) by Pstr_13960 (race Yr9), Pucstr1_11799, and Pucstr1_5472 (race 104E) all belonging to *P. striiformis* only. (Supplementary files S1 and S3).

The 3D Structure of Kiwellin-Like Effectors Significantly Aligns the Crystal Structure of Plant Kiwellin Proteins

Out of 109 modeled candidate effector proteins matching with plant Kiwellins, the structural analysis of the randomly selected candidates showed stable 3D structure as predicted by the Ramachandran plot and SAVES server analysis.

Further, the analysis showed these proteins to show minimal similarity at the sequence level however several residues were found conserved for Chorismate mutase interaction sites. These candidates possess a central β -barrel domain composed of anti-parallel β -sheet similar to the crystal structure of maize (*Zea mays*, PDB Id 6TI2) and kiwi Kiwellin proteins (*Actinidia chinensis*, PDB Id-4PMK) (Fig. 2 and 3). The superimposition of the rust KLE Pstr_13960 with two plants Kiwellin crystal structures 4PMK and 6TI2 showed significant superimposition with Tmalign scores of 0.7882 and 0.7553 (Fig. 3A and B) RMSD score (1–2) thus showing significant similarity to Kiwellin protein-like structure of the rust effectors.

The Rust KLEs are Conserved in the Core Region However Differ in the Loop Regions

The comparative structure analysis of rust KLEs from *P. striiformis* (Pstr_13960), *P. graminis* (Pgr_11348), *P. sorghi* (Psrg_KNZ52496_1), and *M. populina* (Mpop_105821) among themselves showed them to be highly conserved in the central core region but highly diverse outside the core region relating to sequence differences with each Kiwellin members (Fig. 4).

RoseTTA Fold/Alpha Fold Analysis Also Supports the Presence of Kiwellin Protein Fold in Candidate Rust Effectors

The candidate rust effector proteins predicted with Kiwellin fold by PHYRE2 also showed consistent results by RoseTTA Fold and Alpha Fold Colab analysis. More than > 100 candidates analyzed using alpha fold (> 95%) of candidates showed pLDDT scores of 65–83 and the pTMScore in the range of 0.65–0.73 suggesting stable structure predictions (Fig. 5A and B). The structure analysis of Pstr_13960 using Alphafold showed an additional modeled region present at the N-terminus.

Rust KLEs Possess Endonucleases Like Central Predicted Carbohydrate Binding Core Similar to Plant Kiwellins

In addition to the highest structural similarity to plant Kiwellins, rust KLEs also showed the second highest similarity to carbohydrate-binding protein members such as β -1,4-D-endoglucanase 1WC2, Tmalign score 0.596 (Supplementary Fig. 3A and G). The superimposition analysis showed both plant Kiwellin and rust KLEs proteins possess a central double psi beta-barrel (DPBB) carbohydrate-binding core similar to β -1,4-D-endoglucanase 1WC2 and various cell wall degrading domain-containing protein families (Supplementary Fig. 3D–F). However, plant and rust KLEs have a slightly more similar core organization in contrast to the β -1,4-D-endoglucanases that have different α folds β sheets arrangement differentiating between the Kiwellin and carbohydrate-binding protein family domains. Additionally, plant and rust Kiwellins contain three loops outside the carbohydrate-binding central core in addition to major structural differences in α folds β sheets architecture. The Pstr_13960 matched to C-terminal region of Plant Kiwellins hence lacking one α fold and 2 β sheets. However, the rest of the C-terminal showed a similar arrangement in comparison to 1WC2 (Supplementary Figs. 3B and C).

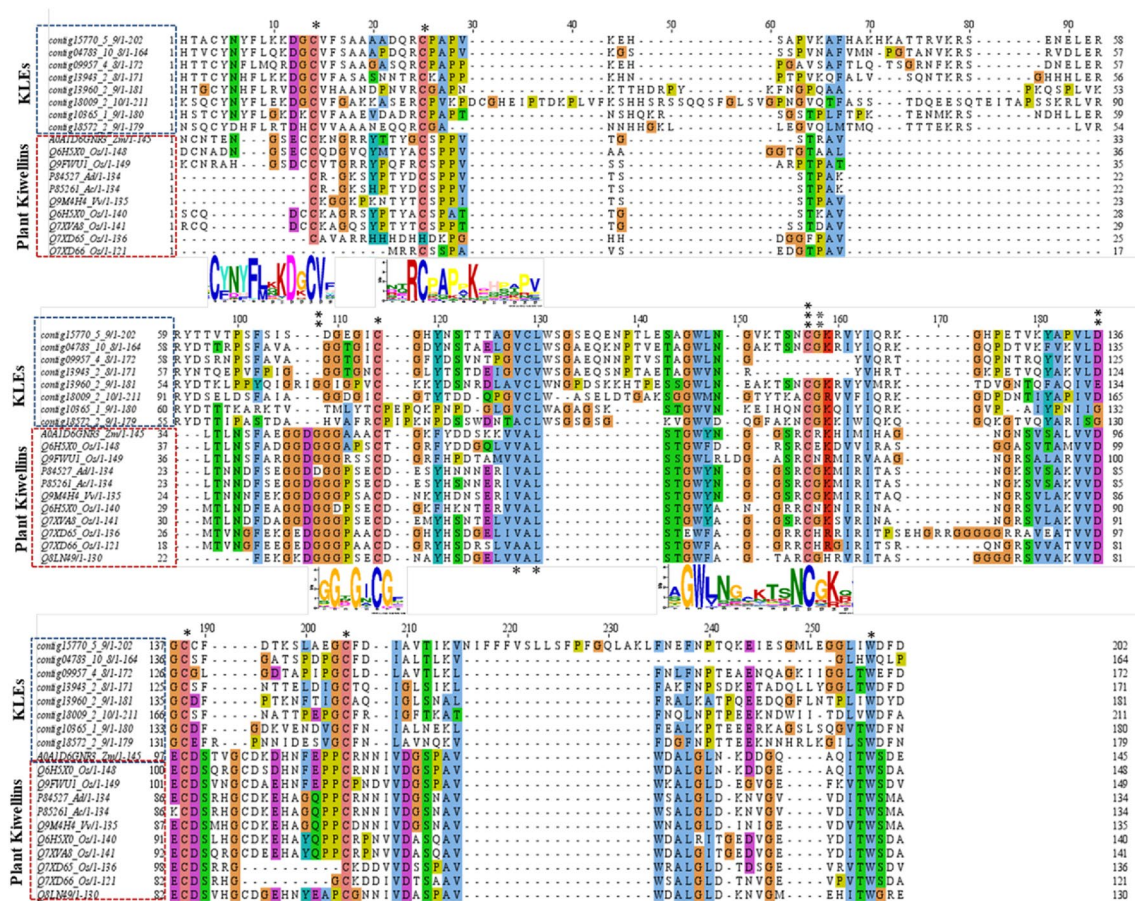


Fig. 2 Sequence analysis showing the conserved region of plant Kiwellin proteins with selected rust KLE proteins. The residues with a single asterisk sign are highly conserved in rust KLEs and plant Kiwellin proteins. The double asterisk represents residues involved in KWL1 or KWLb interaction with the Cmu1 effector whereas the tri-

ple asterisk highlights residues used by KWL1 and KWLb for Cmu1 binding. Top panel of the alignment highlights N-terminal region/less matched region of the rust Kiwellin (mostly rust specific except few residues). The lower two panels represent highly comparatively conserved region of plant and rust Kiwellins (core region)

Rust KLEs are Intrinsically Disordered at the Plant Kiwellin Specific $\beta 1/\beta 2$ and Kissper Regions

During sequence alignment and structure analysis using Phyre2 for proteins, it was interesting to note that most of the modeled rust KLEs matched with plant Kiwellin proteins at the C-terminal than the full region (Fig. 2, 5D). Further analysis of the unmodelled and unmatched region showed rust KLE proteins possess intrinsically disordered regions at the N-terminus after the signal peptide where $\beta 1/\beta 2$, and kissper region is predicted to be present in plant Kiwellins (Fig. 6A and B). The disorder region analysis using SLIDR software predicted these proteins to possess IDR of variable length (Supplementary file S15). Further, the PONDR and IUPred2A softwares also confirmed the presence of disorder regions in the rust KLEs.

Additionally, the Pstr_13960 structure generated by AlphaFold also confirmed a long unmodelled region at the N-terminus (Fig. 5D and 6B). Further structural comparison

of Pstr_13960 with 6T12_A maize Kiwellin showed the presence of a distinctive $\beta 1/\beta 2$ region and Pstr_13960 showed the disordered region at similar N-terminal positions (Fig. 6D). In contrast to unannotated novel Kiwellin candidates that lack the $\beta 1/\beta 2$ region, few of the rust KLE candidates matching with DPBB domain showed the presence of a partial $\beta 2$ region in their structure (Fig. 6G and H).

Intron–exon Organization Analysis Showed Rust Kiwellin Effector Members to be Evolutionarily Conserved Despite the Huge Sequence Variability

Multiple sequence alignment of the fungal Kiwellin fold-like proteins showed diversity in their sequence (Supplementary Figs. 2 and 3). Despite the diversity, these proteins tend to have a core Kiwellin matching region that was significantly conserved when compared to full proteins. The analysis of intron–exon boundaries of selected KLEs from *P. striiformis* showed that they have a similar intron–exon organization

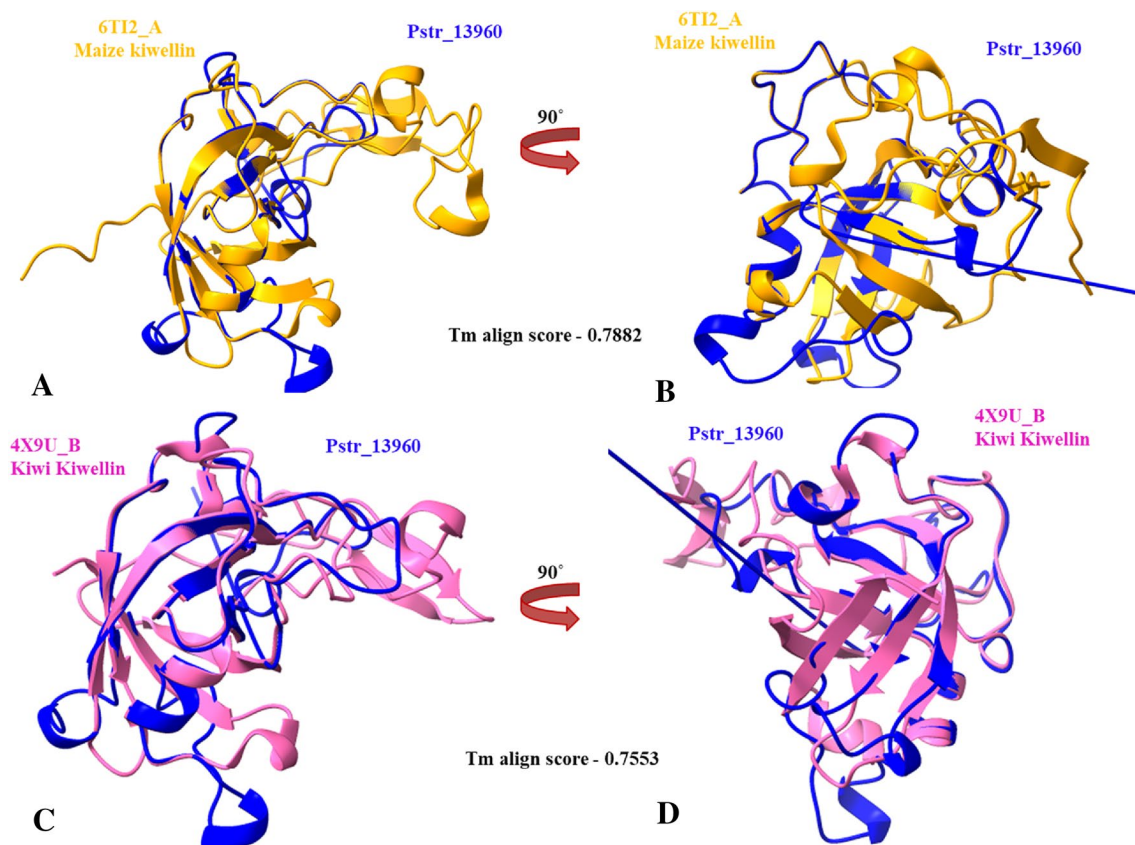


Fig. 3 Structural comparison of Pstr_13960 with plant Kiwelin proteins. **A** and **B**. Structural comparison of Pstr_13960 with two plants Kiwelin protein crystal structures *Zea mays*, PDB Id 6TI2(A) and

(B) and *Actinidia chinensis*, PDB Id-4PMK (C) and (D) showing significant similarity with Tmalign score of 0.7882 and 0.7553

(Supplementary Fig. 4). These randomly selected rust genes were grouped into two categories where one group had nine exons and the other had eight exons. Though the size of the exons and intron more or less remained the same.

Rust KLEs Seem to be Highly Diverse in Sequence Though Several Members Clusters with the Plant Kiwellins Members

The phylogenetic analysis of rust KLEs using Maximum likelihood (ML) methods showed that these proteins could be classified into ten major subgroups showing high diversity among rust fungi. (Fig. 7A) Further, the comparative phylogeny of rust KLE proteins with plant Kiwelin proteins showed grouping into five major groups distinguishing plant and fungal members (Fig. 7B). Though few rust KLE proteins were found to be clustered with plant Kiwelin proteins, signifying more similarity of these rusts KLE members to the plant, and further suggesting an evolutionary relationship between these proteins (Fig. 7B Supplementary Fig. 5). The MrBayes analysis for all rust Kiwelin effectors also classified these proteins into ten

different groups. Further to infer a more precise evolution between rust Kiwellins and plant Kiwellins MrBayes and time tree analysis using the MEGA RelTime method was carried out however no clear inference could be made from the analysis (data not shown).

Rust KLEs are Highly Diverse as Suggested by Orthology and Clustering Analysis

The clustering and orthology analysis of various rust KLEs showed these proteins to be significantly diverse among themselves forming 11 different clusters (Supplementary Fig. 6A). The central common cluster showed 27 proteins from different species where the highest proteins (9) were clustered from *P. coronata*, 6 proteins from *P. sorghi* and 5 proteins were from *M. populina* (Supplementary Fig. 6B and C). Surprisingly *P. striiformis* KLEs seemed to be more diverse as 1 or 2 proteins were present in each of identified clusters (Supplementary Fig. 6A).

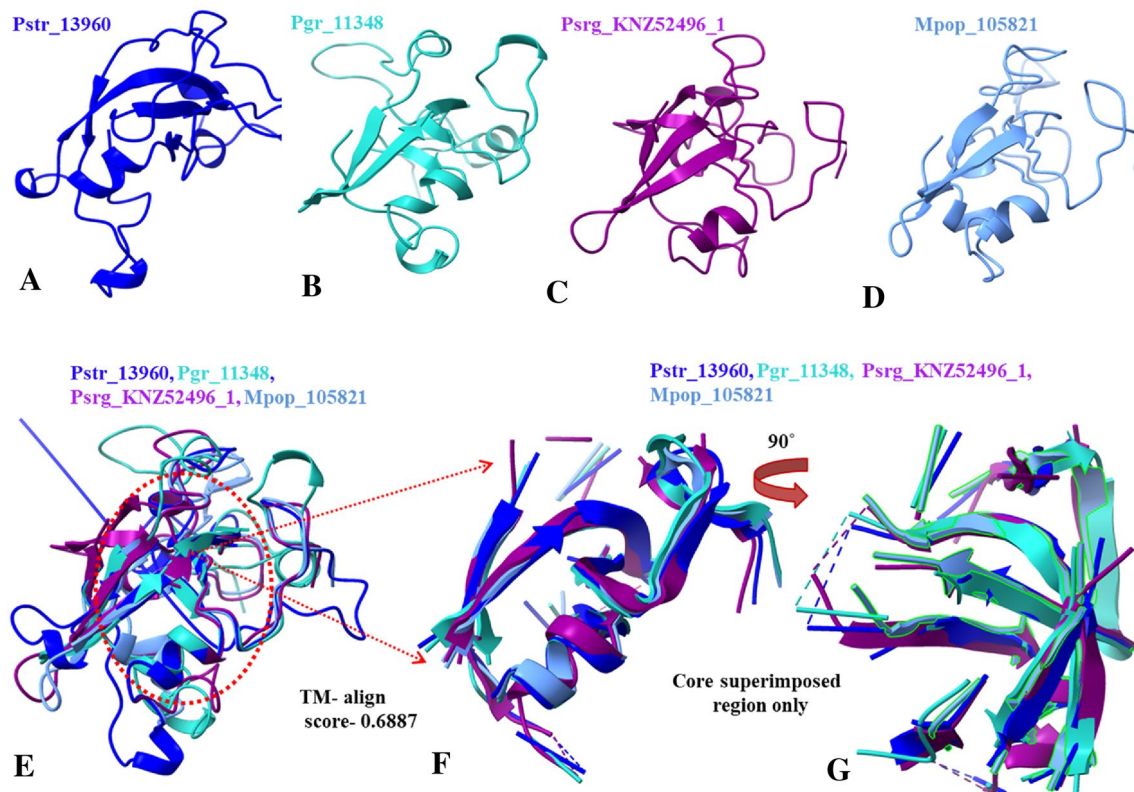


Fig. 4 Structural comparison and core region analysis of the rust Kiwellin effector proteins. (A) *Pstr_13960* (blue) (B) *Pgr_11348* (cyan) (C) *Psrg_KNZ52496_1* (violet) and (D) *Mpop_105821* (cornflower blue) (E) Superimposition of *Pstr_13960*, *Pgr_11348*, *Psrg_KNZ52496_1* and *Mpop_105821* (F) and (G) Conserved core region of *Pstr_13960*, *Pgr_11348*, *Psrg_KNZ52496_1* and *Mpop_105821* in different rotations

***Pstr_13960*, a Candidate Rust Kiwellin Effector Suppresses BAX-Induced Cell Death, Localizes to the Chloroplast in *N. benthamiana*, and is Expressed in Diverse Conditions.**

The *Pstr_13960*, one of the *P. striiformis* Yr9 race rust KLE showed the highest modeling confidence (97.8%) and 30% sequence similarity therefore was selected for functional characterization. The transient expression studies of *Pst_13960* in *N. benthamiana* using agroinfiltration did not show any cell death-inducing phenotype; however, the *Pst_13960* was able to suppress BAX-induced cell death in *N. benthamiana* leaves when BAX was agroinfiltrated 24 h after *Pstr_13960* (Fig. 8A and B). The subcellular localization analysis in *N. benthamiana* showed *Pstr_13960* to be primarily found to localize in the chloroplast (Fig. 8C). Further the expression analysis of *Pstr_13960* homologs in various host-infected samples showed it to express in diverse environmental conditions such as flowering, different leaf stages, stages of infection cycles in different geographical locations. (Supplementary Fig. 7).

N-terminus Encompassing Intrinsically Disorder Region Putatively Contain Chloroplast Targeting Signal of *Pstr_13960*

To identify the chloroplast targeting region of *Pstr_13960* various deletion fragments were created (Fig. 9A). The full *Pstr_13960* gene encoding protein primarily localizes to the chloroplast (Fig. 9B1). The removal of the signal peptide encoding region also did not affect the chloroplastic location of *Pstr_13960* (Fig. 9B2). Further, the deletion of the Kiwellin encoding region also did not change the location of *Pstr_13960* (Fig. 9B3). Surprisingly the expression of the Kiwellin core matching region alone after the deletion of the N-terminal region including the disordered region showed it to be primarily localized to the cytoplasm and nucleus. (Fig. 9B4).

The Kiwellin Core Matching Region of *Pstr_13960* Alone is Sufficient to Suppress the BAX-Induced Cell Death

To further characterize the Kiwellin matching region *Pst_13960_Kiwi* was also expressed in *N. benthamiana*

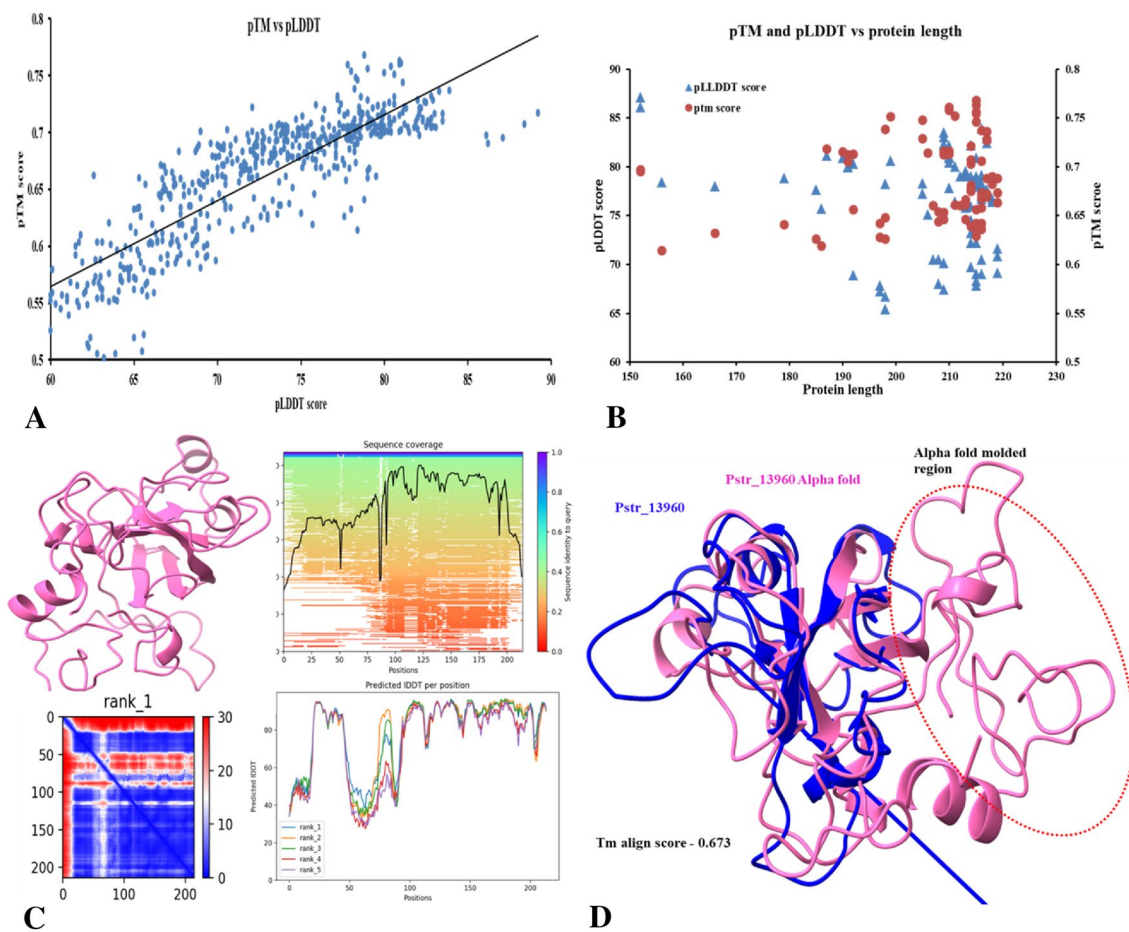


Fig. 5 Alpha Fold analysis of Kiwellin matching rust candidate effector proteins. **A** pTM and pLDDT score comparison of candidate proteins showing significantly stable predicted structures. **B** pTM, pLDDT score vs candidate protein length comparison for analyzing the effect of protein length on the stable structure prediction. **C**

Alpha Fold predicted structure analysis of Pstr_13960 showing complete predicted structure, sequence coverage, quality of the structure, and predicted IDDT score per position. **D** Structural comparison of Pstr_13960 Phyre2 with Pstr_13960 Alpha Fold showing an additional modeled region of Pstr_13960 by Alpha Fold at N-terminus

using BAX cell death suppression assay. The *Pst_13960_Kiwi* also suppressed the BAX-induced cell death however its location was changed from chloroplast to the nucleus and cytoplasm when analyzed for subcellular localization (Fig. 10).

Molecular Docking Shows Rust KLEs Can Bind with Plant Chorismate Mutase Proteins (CMs)

The plant Kiwellins have been reported to interact with fungal Chorismate mutases (CMs) effectors using side loops and $\beta 1/\beta 2$ regions. The structural comparison of Pstr_13960 with maize Kiwellin showed that in addition

to the central core, three loops involved in the interaction with chorismate mutases are also conserved in rust Kiwellin effectors (Fig. 11A)., however, the $\beta 1/\beta 2$ region was predicted disordered in the majority of rust candidates using Alpha Fold analysis (Fig. 6G).

We performed the protein–protein docking of the maize, wheat, and tobacco CM with rust Kiwellin proteins. The results showed rust KLEs may bind plant CMs proteins using these loops (Fig. 11B–D). The interaction analysis performed with all 5 different rust effectors proteins with different CMs proteins of maize, tobacco, and wheat, showed significant binding.

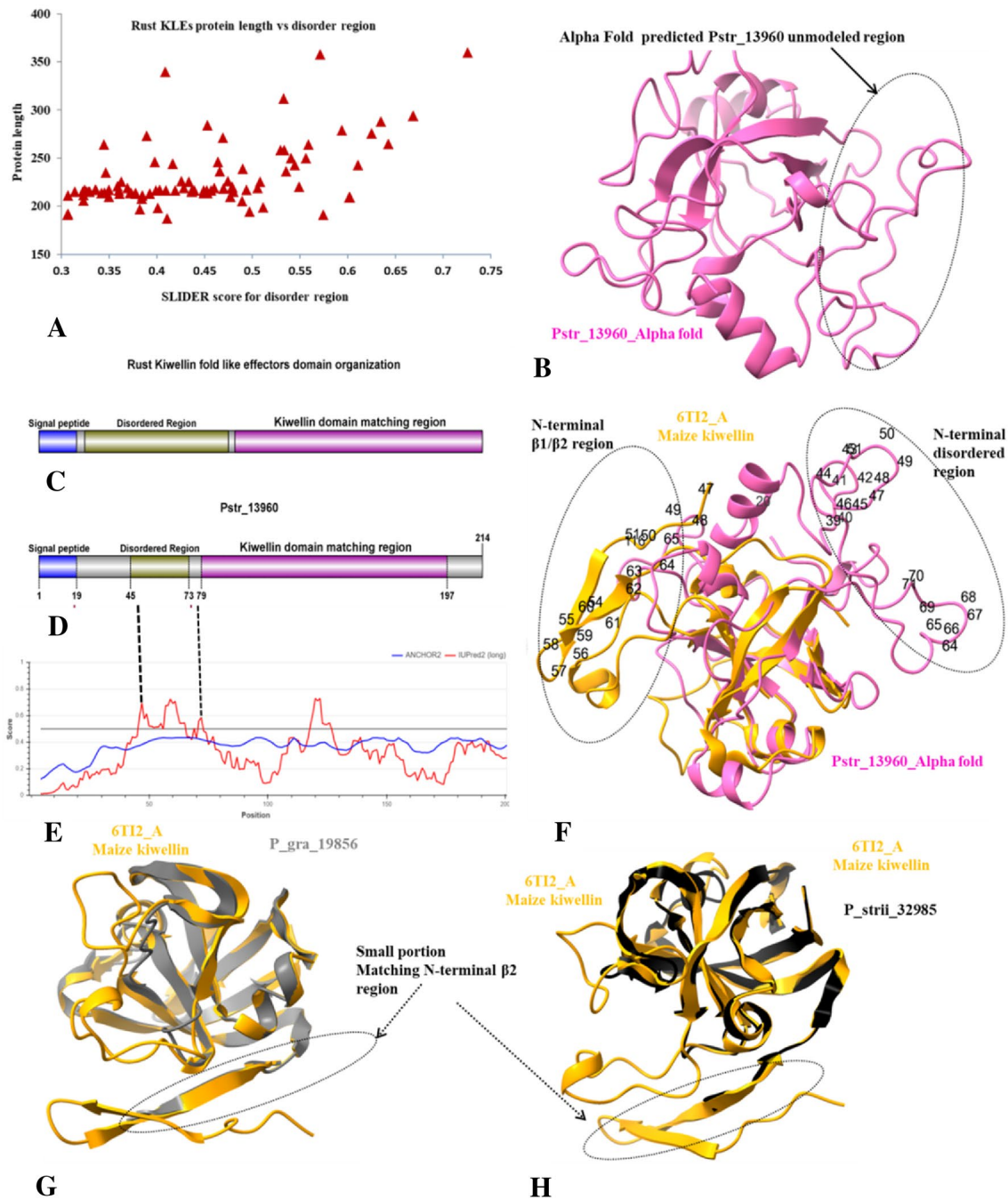
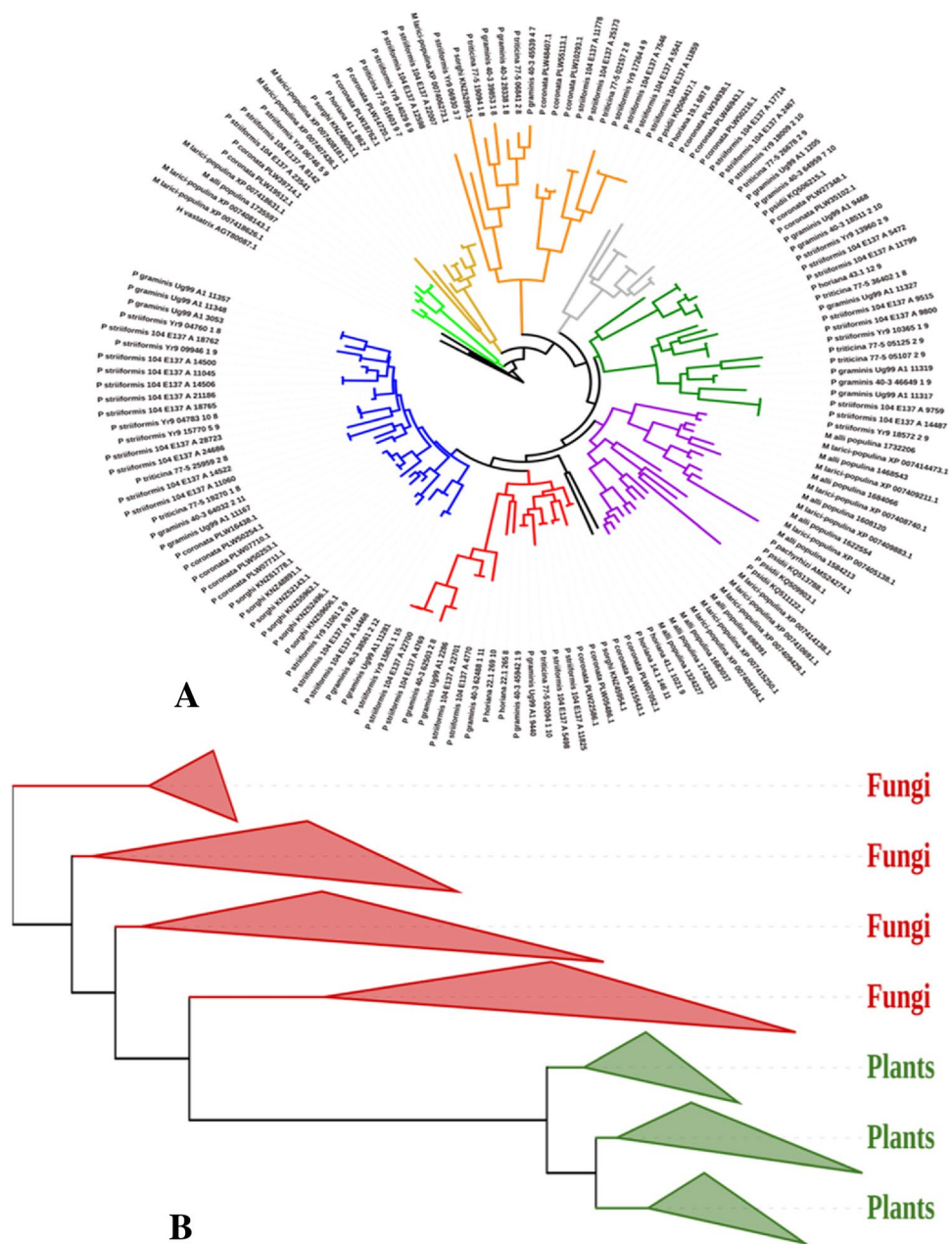


Fig. 6 Disorder prediction and domain organization of the rust KLEs. **A** Disordered region prediction comparison of protein length and disordered region in the rust KLEs using SLIDER software. **B** Confirmation of the unmodeled region of Pstr_13960 using Alpha Fold analysis. **C** General rust KLEs domain organization showing signal peptide disordered region and DPBB core region. **D** Structural comparison of Pstr_13960 with 6TI2_A maize Kiwellin highlight-

ing plant $\beta 1/\beta 2$ region and rust effector unmodeled region however at similar N-terminal positions. **E** Pstr_13960 domain organization possessing N-terminal disordered region after signal peptide. **F** Intrinsic disorder region prediction of Pstr_13960 using IUPred2A software. (**G** and **H**) DPBB domain containing two rust Kiwellin effectors was found to show slight similarity to the $\beta 1/\beta 2$ region

Fig. 7 Phylogenetic analysis of rust KLEs proteins. **A** Phylogenetic analysis of rust KLEs classified these proteins into ten different groups. Group 1 (blue), group 2 (red), group 3 (violet), group 4 (green), group 6 (light grey), group 7 (orange), group 8 (yellow), group 9 (light green), group 10 (black). **B** Selected plant Kiwellin proteins (model plant species) were used for comparing the Kiwellin proteins. The rust and plant proteins formed separate clusters surprisingly few rust KLEs clustered more closely to plant Kiwellins. Plants Kiwellins (green), rust KLEs (red)

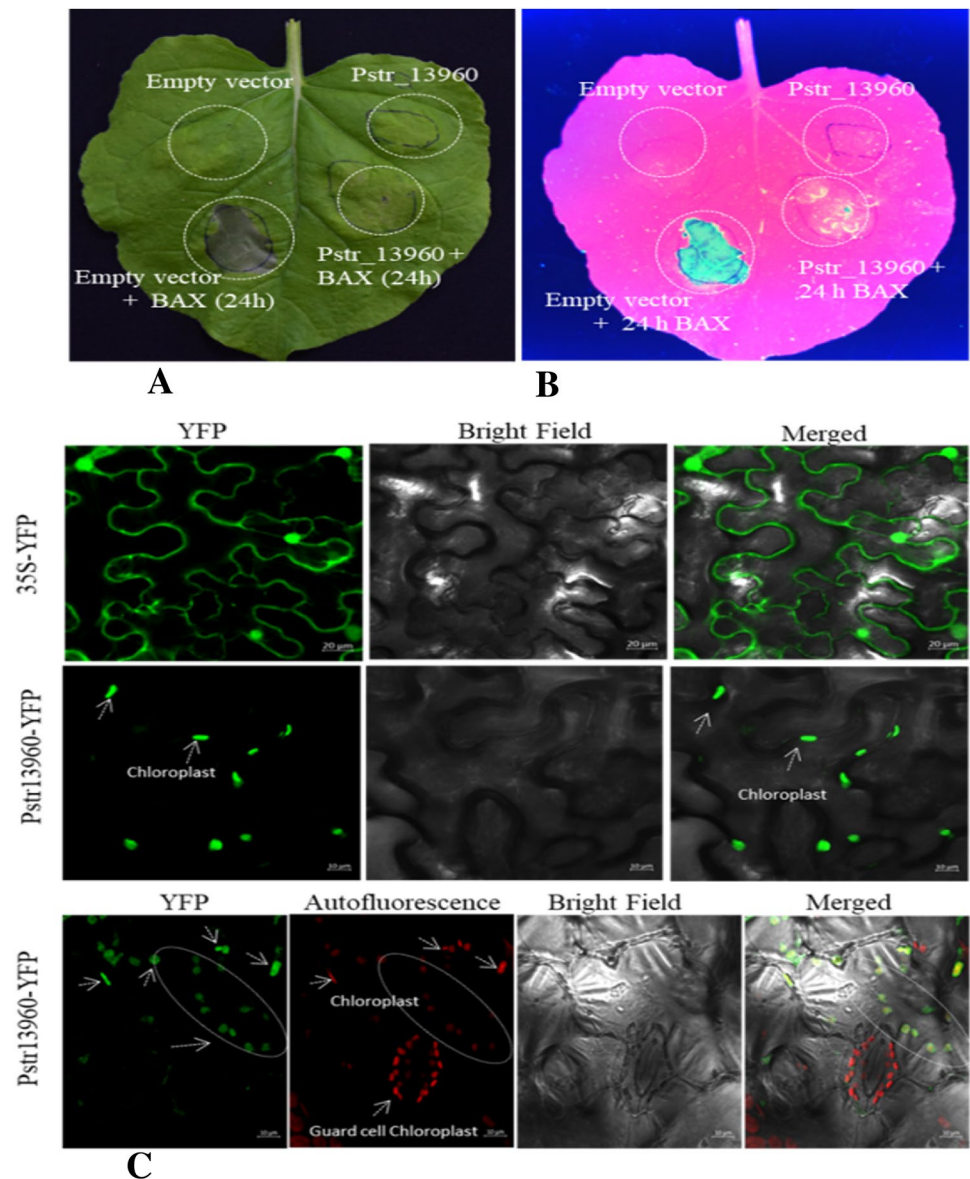


DPBB Domain Matching Kiwellin Fold-Containing Effector Proteins are Also Present in Diverse Fungal Species However Largely Associated with Class Basidiomycetes.

The identification of Kiwellin effectors in other plant pathogens using rust candidate proteins as a query did not give any significant results however using plant Kiwellins proteins as a query returned several hits belonging to different fungal species (Maximum hits selected 500) (Fig. 12A). The

domain annotation of these proteins showed a match to the DPBB domain. Further, the secretory, effector, and structure modeling analysis of 500 candidates returned 107 proteins with Kiwellin-like-fold with variable confidence. Further analysis of the results showed the Kiwellin-like fold containing candidate effectors may not only restricted to plant pathogens moreover also present in non-pathogen wood decaying, symbiotic, and animal-infecting yeast species. Further analysis also showed that these candidates are largely associated with class basidiomycetes (Fig. 12A–G).

Fig. 8 BAX-induced cell death suppression assay and localization of *Pstr_13960*. **A** *N. benthamiana* leaf showing *Pstr_13960* suppresses the BAX-induced cell death. **B** The leaf as in figure A under image analyzer (RGB color) (**C**) Localization of *Pstr_13960* in *N. benthamiana* using agroinfiltration showed its location in chloroplasts



Discussion

Functional annotation of unannotated candidate effector proteins has proved to be the most prominent hurdle in assigning their role in plant-pathogen interaction. In the present study, we have performed comparative secretome and computational structural genomic studies to identify conserved known protein folds in unannotated effectors to define their putative function in host manipulation. We performed this study on diverse plant pathogens ranging including oomycetes and rust fungi. Variable unannotated secretory proteins were identified in the studied species. Biotrophs followed by hemibiotrophs generally encode a large number of novel proteins in contrast to the necrotrophs that require the least amount was also reflected in our study (Supplementary Table 2). As expected, we observed these candidates

share minimal similarities at the primary sequence level, as well as at a structural level (> 20% sequence similarity, 60% modeling confidence, Table 1). However, the 3D structures of several candidates showed significant similarity to conserved known effector families that are known to play a role in host defense manipulation. Among these, the microbial nucleases and RNA binding fold-containing effectors were worth considering as these have been reported for several powdery mildew species [8, 12, 44]. These effectors are known to attenuate host immunity by interfering with the host RNA degradation system. The similarity of RNA binding folds to unannotated effectors suggests the effectors are directly involved in host manipulation and have evolved leading to divergence from the original family. Furthermore, a recent Alpha Fold-based analysis of the secretome of the *B. graminis* f. sp. *hordei* showed the presence of 426

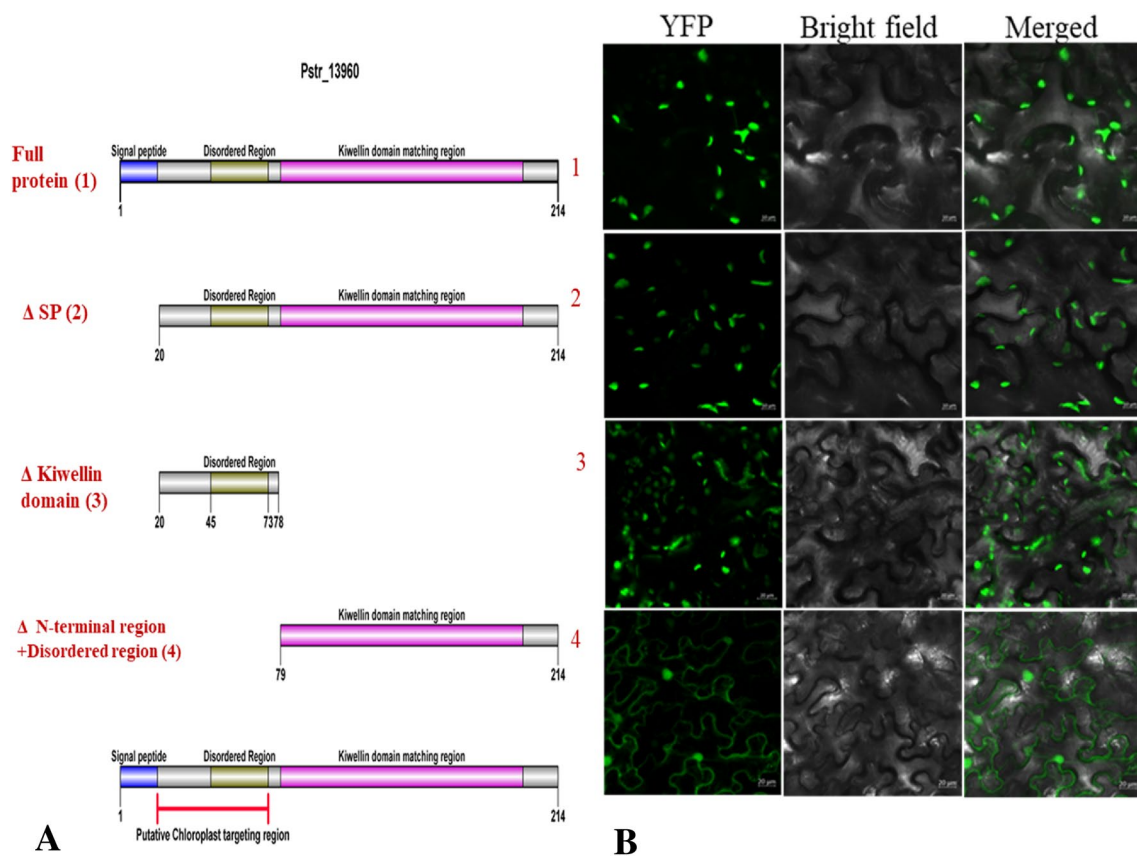
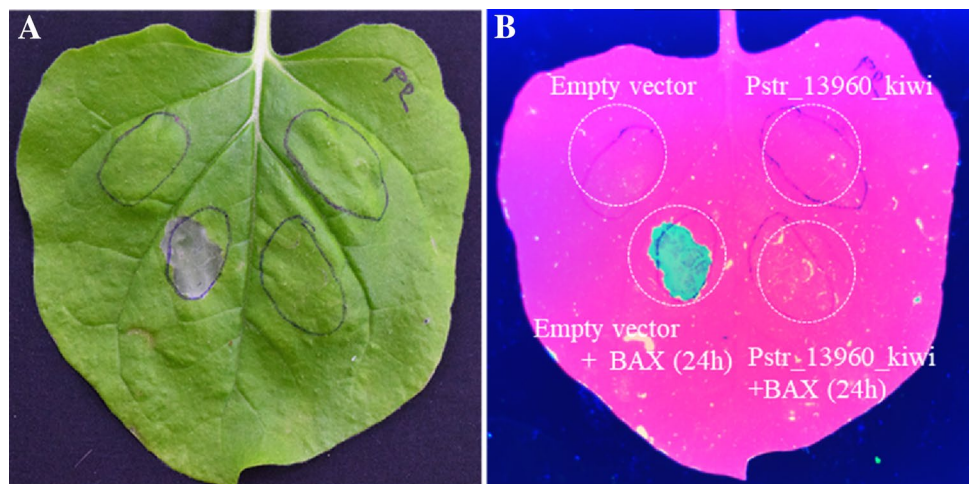


Fig. 9 Various deletion fragments of *Pstr_13960* regions targeting chloroplast, plasma membrane, and nucleus in *N. benthamiana*. **A** Representation of deletion of various regions of *Pstr_13960* for analyzing the chloroplast targeting region. **B** Subcellular localization analysis after various deletions. A region near signal peptide

and disordered region putatively encode chloroplast targeting region as Kiwellin core matching region alone localized to cytoplasm and nucleus. 1. Full protein, chloroplast. 2. Without signal peptide, chloroplast. 3. Without the Kiwellin region, chloroplast. 4. Kiwellin core region only, cytoplasm and nucleus

Fig. 10 Cell death suppression assay for *PStr_13960_kiwi* using agroinfiltration. **A** *N. benthamiana* leaf showing BAX-induced cell death suppression. **B** The leaf is in figure A under the image analyzer (RGB color)



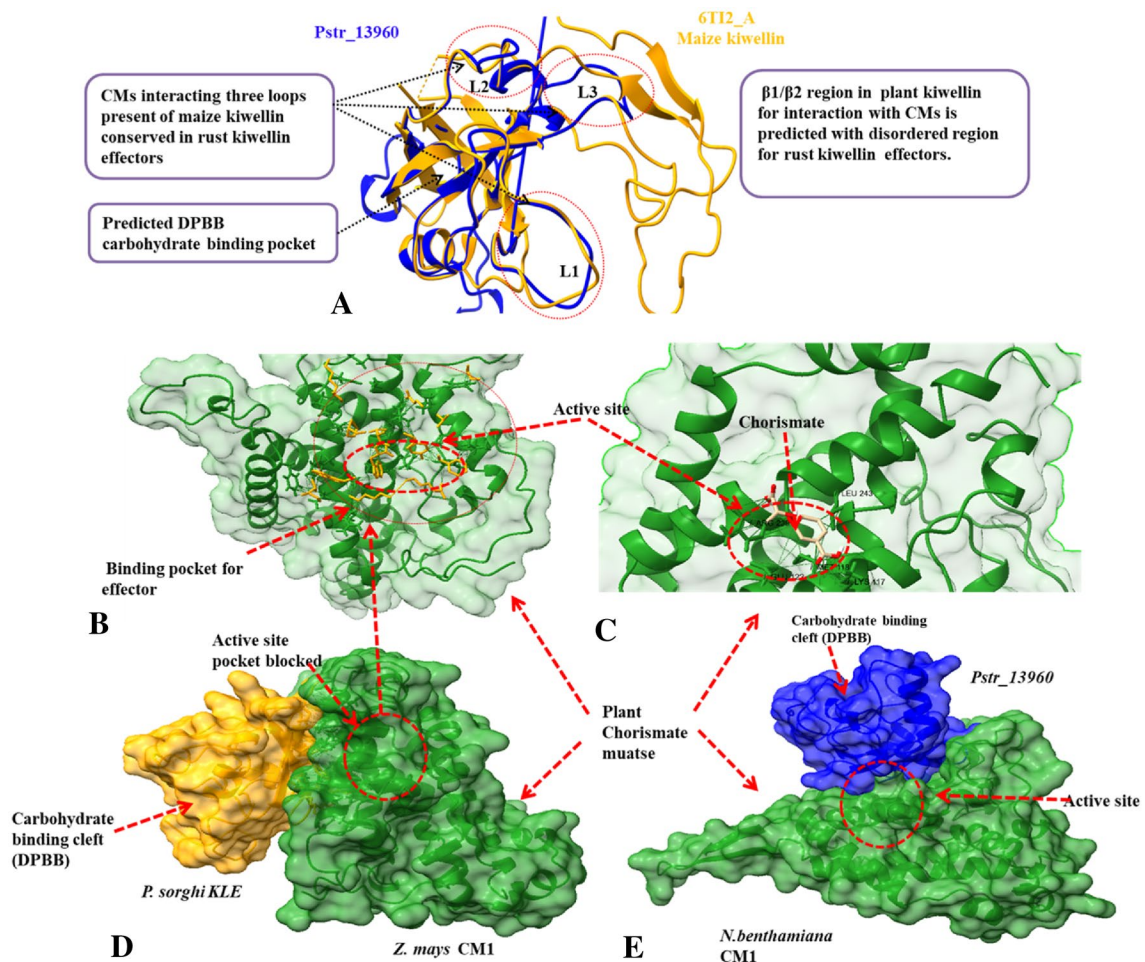


Fig. 11 Molecular docking of rust KLEs with plant Chorismate mutases. **A** Comparison of chorismate mutase interacting sites of maize Kiwelin sites (with rust Kiwelin showing revealing rust effector possesses all the three loops (L1, L2, and L3) however $\beta 1/\beta 2$ region was predicted disordered and hence could not be aligned to

plant kiwells. **B** Interacting sites of Kiwelin effectors with maize chorismate protein (CM1) showing binding pocket region. **C** The binding of *P. sorghi* KNZ52496 covers the chorismate binding pocket of maize CM1. **D** Binding of Pstr_13960 covers the chorismate binding pocket of *N. benthamiana* CM1

RNases-like effectors nearly comprising 43% of the total secretome signifying the importance of extreme expansion of this fold containing effectors in powdery mildew fungi [19].

Several lytic polysaccharide monooxygenases (LMPOs) and Gamma-Crystallin-like fold-containing members were found in oomycetes where the former is involved in oxidative degradation of biopolymers such as chitin and later is reported to perform in diverse biological processes. LMPO has been reported to be involved in host manipulation in diverse pathogens such as plants, animal fungi, and oomycetes [45, 46]. *P. infestans* LMPO members have been proposed to function in degrading host cell walls for facilitating host entry moreover oxidizing pectin-related oligosaccharides for eluding host PTI [47]. In addition to the RNases and LMPOs, several other candidates matched with RxLR

effectors, knottins fold, Thaumantoin, and Smp-1-like folds in the studied pathogens, where all of these families known to be associated with host defense manipulation implying highly diverged distantly related effector family members may contain conserved folds to retain original function.

Among all the candidates containing pathogen effectors matching to plant Kiwells was intriguing. Looking at the function of Kiwelin proteins in host defense, the presence of Kiwelin-like secretory proteins in rust pathogens was very surprising as both host defense and pathogen virulence arsenal contain the same family. Though the study carried out by Han et al., 2019 also reported the presence of Kiwelin-like proteins in eukaryotes including a few fungal species having DPBB domain, no detailed study was carried out to analyze their presence in secretome or function as an effector/virulence factor [21].

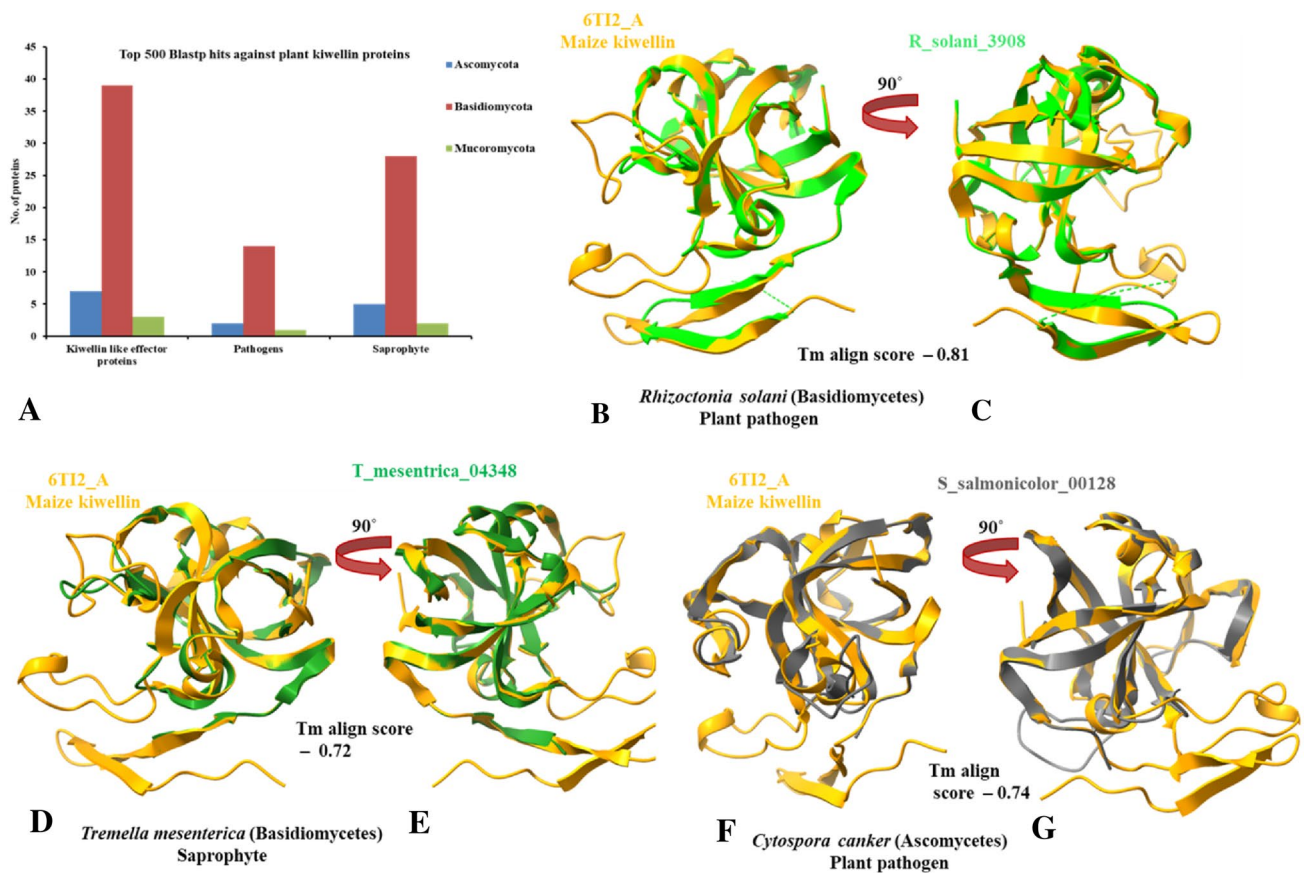


Fig. 12 Identification of Kiwellin-like effectors in plant fungal pathogens. **A** BLASTp against NCBI-nr database (fungi only) using plant Kiwellin as the query. Top 500 hits were analyzed for secretory, effector, and analyzed for Kiwellin fold. Basidiomycetes were the high-

est among the found species. **B–G** Structural comparison of selected Kiwellin fungal effectors with maize Kiwellin protein showing significant conservation and presence of $\beta 2$ from $\beta 1/\beta 2$ region

Further exploring the presence of these candidates in rusts we found a diverse number of Kiwellin effectors (minimum 1 and a maximum of 26 proteins per species, Table 2) with high diversity suggesting the diverged functions of these candidates. Such diversity in sequence and numbers of these proteins could be explained by two possibilities. First, the species studied here belong to different geographical locations, and as a result, may have expanded the gene family due to variable selection pressure and further diversification of these effectors. The second possibility is the use of different sequencing platforms for sequencing the rust genomes as a result of a partially assembled genome. The highest number of KLEs were found in two recently sequenced genomes *P. striiformis* 104E137A and *P. coronata*, where both have been sequenced by long-read sequencing technologies [48, 49]. The fewer KLEs found in all other previously sequenced genome assemblies could be the result of short-read sequencing technologies or incomplete assemblies of these genomes. Considering the results of two advanced long-read sequencing technologies assemblies we speculate that the rust genome may encode

several KLEs showing signs of expansion, however, many of these members could not be identified due to short-read fragmented assemblies of the other rust species (Table 2).

In addition to the presence of KLEs in rust fungi, we also found these candidates in several plant pathogen and non-pathogen wood-decaying basidiomycetes. The presence of Kiwellin-like effectors in non-pathogenic fungi suggests that these members have gone through divergent evolutionary pressure and evolved to perform a broader function in addition to the typical host defense manipulation roles like effectors. Moreover, the Kiwellin proteins are known to possess a core double psi beta-barrel (DPBB) domain that matches with several other protein families with expansion-like features, and sugar-binding proteins [21, 22, 24]. Though structural modeling significantly distinguished proteins with Kiwellin-like fold and sugar-binding fold-containing proteins of these fungal pathogens. The DPBB core of these effectors still may play role in carbohydrate binding in addition to performing typical Kiwellin-like function. Earlier carbohydrate binding assay for plant Kiwellin proteins showed these proteins bind but lack enzymatic activity [24].

No fungal Kiwellin effectors have been analyzed for carbohydrate binding activity, hence the role of fungal Kiwellin effectors as the additional role in carbohydrate binding can not be eliminated.

The Kiwellin proteins in plants are known to affect the activity of the Cmu1 effector by inhibiting its binding to host chorismate proteins so that enough salicylic acid (SA) could be produced to defend against pathogen infection, especially biotrophs. In this scenario, the role of KLEs could be highlighted by the alternate strategy of fungal pathogens to halt SA synthesis. As plant Kiwellin captures fungal CMs allowing free plant CMs to produce SA synthesis. In response to this, as a counter-strategy, the Kiwellin effectors may bind to plant CMs to suppress SA production and promote infection. The docking analysis showed rust KLEs can interact to plant CMs further strengthening the possibility of interaction of rust KLEs with plant CMs. The residues involved in the CM interaction are also conserved in the rust KLEs when compared with maize KWL1 and KWL1b (Fig. 2). The Pstr_13960 and Kiwellin matching region (Pstr_13960_kiwi) also showed BAX-induced cell death suppression showed its potential effector activity. Moreover, we find Kiwellin-like effectors in several plant pathogens including smut fungi, *Rhizoctonia solani*, members of *Microbotryum* species, *Cytospora leucostoma*, and several other species, suggesting that fungal pathogens might have evolved these effectors as an alternate strategy, in the course of evolution to fail plant Kiwellin proteins mediated defense response. Though, further interaction studies may validate the binding of KLEs with plant CMs to prove the theory.

Further, the plant chorismate mutase primarily localizes in the chloroplast and the cytoplasm. The Pstr_13960 and Pstr_13960_kiwi also show the same location as predicted in subcellular localization studies suggesting the interaction of Pstr_13960 with plant CMs is feasible. Though plant Kiwellins possess three distinct loops and an N-terminal $\beta 1/\beta 2$ region to perform an interaction with CMs [21, 24]. We observed rust KLEs to possess three loops but lack the complete $\beta 1/\beta 2$ region, instead possess disordered regions at similar positions producing loops (Fig. 12).

The presence of the N-terminal disordered region at $\beta 1/\beta 2$ and kissper corresponding regions in the 3D structure of rust KLEs raises several questions. whether the region was modeled properly or it truly contains the disordered region. Though sequence-based disordered analysis could not predict the IDR in kissper region of the Pstr_13960. Several crystal structures of recently analyzed plant Kiwellin proteins showed the kissper-containing region could be highly unstructured [20, 50]. The kissper region also has conformational flexibility, and possibly disordered conformations in the crystal structure and solution form, suggesting kissper region could be predicted as disordered due to its dynamic structure.

However, the prediction of the $\beta 1/\beta 2$ region as disordered could be of special interest as it is known to interact with CMs effector [24]. The presence of the IDRs in the effectors is predicted to impart diverse functions to the proteins. In the case of bacterial effector proteins, the IDRs are associated with several functions such as translocation, secretion, and interaction with multiple candidates. The presence of IDR is also a common structural characteristic for several RxLR and WY effectors proteins [51, 52]. The study carried out by Shen et al., 2017 also showed that RxLR predicted region in these oomycetes effectors is preferably disordered [53]. Moreover, the mutation in the disordered region also leads to loss of avirulence activity for PsAvh18 and PcAvh207 effectors further suggesting the importance of the disordered region in effectors proteins [53, 54]. Therefore it is likely that the kissper region in rust KLEs might have evolved in the transient peptide. Though the N-terminal region (after signal peptide and before predicted IDR/putative $\beta 1/\beta 2$ region) was also comparatively smaller than the average reported chloroplast transient peptides (20–45 amino acids, 25AAs), we can not rule out the involvement of other IDR portions (putative $\beta 1/\beta 2$ region 45–73 (28AAs) in translocation [42]. In addition to their role in translocation, disordered regions can also enable proteins to interact with multiple partners transiently. Therefore, the evolution of the $\beta 1/\beta 2$ region into the IDR region could be associated with the ability of these effectors to interact with multiple host proteins to attenuate host immunity.

Conclusions

The Kiwellin proteins have been known recently for their role in the plant defense against pathogens. These proteins are present in several eukaryotic species. In this study, we report the presence of these proteins in the fungi and predicted them to act as potential candidate effectors in rust fungi. We propose that the DPBB domain containing Kiwellin matching effectors is widely present in eukaryotes including plant pathogens and non-pathogens. Plant pathogens such as rusts might have evolved several novel Kiwellin effector candidates that are diverged from the original DPBB domain family. We also predicted that rust KLEs may act as effectors probably by interacting to plant CMs as a counter-strategy when host Kiwellin proteins attempt to bind to rust CMs. As Kiwellin and Cmu effectors are widely present in plant pathogens. The evolution of Kiwellin proteins as effectors could be an alternate strategy of fungi to avoid plant Kiwellin proteins mediated host defense when chorismate mutase effector mediated host manipulation mechanism becomes ineffective. The engineering of host Chorismate mutase targeted by Kiwellin effectors can be used to develop durable cultivars against these pathogens.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12033-023-00717-y>.

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Author Contributions Conceptualization: TRS and RJ. Data curation and experiments: RJ. Data analysis and writing first draft: RJ, SR, HD, KK, HR, HS, RD. Funding acquisition: TRS. Investigation: RJ. Methodology and writing final draft: TRS and RJ.

Data availability All data generated or analyzed during this study are included in this article.

Declarations

Conflict of interests The authors declare no conflict of interests.

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