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Target prediction of potential candidate miRNAs from *Oryza sativa* to silence the *Pyricularia oryzae* genome in rice blast

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Rice (*Oryza sativa*) is a staple food for billions of people across the globe, that feeds nearly three-quarters of the human population on Earth, particularly in Asian countries. Rice yield has been drastically reduced and severely affected by various biotic and abiotic stresses, especially pathogens. Controlling the attack of such pathogens is a matter of immediate concern as yield losses in rice crops could deprive millions of lives of nourishment worldwide. *Pyricularia oryzae* is one such pathogen that has been considered the major disease of rice because of its worldwide geographic distribution. *P. oryzae* belongs to the kingdom fungi, that causes rice blast ultimately adversely affecting the yield of the rice crop. Keeping in view this alarming scenario, the present study was designed so that the identifications of genome-encoded miRNAs of *Oryza sativa* were employed to target and silence the genome of *P. oryzae*. This study accomplished the computational analysis of algorithms related to miRNA target prediction. Four computational target prediction algorithms i.e., psRNATarget, RNA22, miRanda, and RNAhybrid were utilized in this investigation. The consensus among target prediction algorithms was created to discover six miRNAs from the *O. sativa* genome with the conservation of the target site fully evaluated on the genome of *P. oryzae*. The discovery of these novel six miRNAs in *Oryza sativa* paved a strong way toward the control of this disease in rice. It will open doors for further research in the field of gene silencing in rice. These miRNAs can be designed and employed in the future as experimentation to create constructs regarding the silencing of *P. oryzae* in rice crops. In the future, this research would be surely helpful for the development of *P. oryzae* resistant rice varieties.

Keywords *Pyricularia oryzae*, miRNA, *Oryza sativa*, Target prediction, Computational

Rice feeds over half the world's population, acting as a vital source of calories and energy, particularly in Asia and Africa[1]. Its reliable harvests and versatility ensure food security for millions, preventing malnutrition and promoting stability[2]. Rice is a valuable commodity, traded internationally and influencing global markets. Countries like Thailand, Vietnam, Pakistan, and India are major exporters, generating revenue and foreign exchange through the rice trade[3]. Rice isn't just for culinary delights, its byproducts, like bran and husk, find uses in animal feed, fuel, building materials, and even cosmetics[4]. Asia dominates the world's rice production and consumption, with a staggering 90% share, because of its warm and humid climate[5–8]. In Pakistan, it serves as a major source of food, income, and foreign exchange through export. Based on the data from the Economic Survey of Pakistan (2017–18), it is clear that the land devoted to growing rice has experienced a significant increase of 6.4%, rising from 2.74 million hectares in 2016–17 to an astonishing 2.89 million hectares. In addition, the production of rice has also seen a remarkable surge of 8.7%, resulting in an impressive yield of 7.44 million tons compared to 6.84 million tons. Rice output is expected to increase from 8.4 million tons to 9.3 million tons in 2021–22. The

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total production area of rice in Pakistan was 3,041,000 hectares, as per the Food and Agriculture Organization of the United Nations (FAO)[6,9–12].

Pyricularia oryzae, also known as *Magnoparthe oryzae*, is a plant pathogenic fungus that poses a significant threat to rice crops worldwide[13]. The genomic information of *Pyricularia oryzae* varies among strains, and the fungus has been extensively studied through genome sequencing efforts. While the genome size can differ between isolates, researchers have identified key genomic features associated with pathogenicity, including regions housing virulence factors and effectors. According to data accessed from NCBI database (15 Jan. 2024) the genome of *Pyricularia oryzae* consists of 13,860 genes and 479,515 proteins. The genomic data has provided valuable insights into the molecular mechanisms underlying the fungus's ability to cause rice blast disease and interact with its host plant.

Pyricularia oryzae, is classified under the *Sordariomycetes* class in the Ascomycota phylum[14]. *Pyricularia oryzae* directly infects panicles and developing grains, causing them to become discolored, shriveled, and sterile, leading to substantial yield losses[15]. In severe cases, the entire panicle can be blighted, resulting in no grain formation at all. *Pyricularia oryzae* infects leaves, causing characteristic elliptical lesions that disrupt photosynthesis. This reduces the plant's ability to produce sugars for grain development, impacting yield. Severely infected leaves fall off prematurely, further limiting the plant's photosynthetic capacity and grain filling. Under favorable conditions for the fungus, rice blast can devastate entire fields, causing yield losses of up to 100%. Even in milder cases, yield reductions of 10–30% are common, posing a significant challenge for rice farmers and global food security. On the nutritional front, infected rice can show reduced protein content, a crucial nutrient for millions who rely on this staple. Blighted grains also tend to accumulate harmful mycotoxins, posing health risks.

This study aims to explore the potential of genome silencing approach in combating Rice blast and pave the way for a safer and more sustainable future. The utilization of genome silencing technology involves the application of RNAi to effectively cleave dsRNA into miRNAs that bind to mRNA, thereby impeding the translation of mRNA into protein[16]. The MicroRNAs also known as miRNAs, are small non-coding RNA molecules that play a crucial role in genome silencing. The miRNAs are approximately 21–25 nucleotides in length and are generated from hairpin-shaped RNA precursors. The miRNAs bind to complementary sequences of mRNA, which prevents mRNA from being translated into proteins[17].

This study aims to predict *Oryza sativa* encoded miRNAs by employing in-silico approach. Four unique plant-based miRNA target prediction algorithms were utilized in this study. The miRNAs predicted by consensus of these four algorithms would be short-listed which can have the potential to silence the genome of *Pyricularia oryzae*. By the use of computational tools, we can predict the sites in the pathogenic fungal genome which would help control Rice blast. The predicted *Oryza sativa* miRNAs were further evaluated by estimation of free-energies, prediction of their secondary structure, site conservation analysis and also construction phylogenetic trees to study the evolutionary relationships among them.

Materials and methods

Extraction of miRNAs from miRBase

The first step was to collect data in the form of miRNAs from the public database. The miRNA data was collected from a primary data acquisition source which was miRbase https://www.mirbase.org/. Overall, 100 mature sequences of osa-miRNAs belonging to *Oryza sativa* were extracted from miRBase which were extracted and copied as separate files (http://www.mirbase.org/).

Retrieval of fungus genome from NCBI

The complete genome of *Pyricularia oryzae* with size 6358bps was retrieved from NCBI (Accession no. KY509032.1) and copied in another separate FASTA file (https://www.ncbi.nlm.nih.gov/).

Sequence analysis and ORF prediction of fungus

The genome sequencing of *Pyricularia oryzae* (*P.oryzae*) was done to explore various open reading frames (ORFs). The ORFs prediction of whole *Pyricularia oryzae* was executed by exploiting CLC Genomics Workbench (v23). The tools helped in correctly predicting ORFs and the order of nucleotides in *Pyricularia oryzae* genome highlighted in different colors to help in identification.

Target prediction

This research work was based on insilico approach of employing collections of *Oryza sativa*-encoded miRNAs to identify potential targets for silencing the genome of *P. oryzae.* A combination of four different target prediction algorithms, including RNA22, psRNATarget, RNA hybrid, and miRanda were exploited (Fig. 1). Predicting the targets for silencing the genome of *Pyricularia oryzae* involves identifying key miRNAs when silenced, could disrupt the fungus's growth and pathogenicity. Four miRNAs target prediction algorithms were utilized and Table 1 below shows the web sources and parameters opted for this prediction.

Target prediction by psRNATarget

psRNATarget is a computational tool for predicting the interactions between microRNA (miRNA), and its target messenger RNA (mRNA). This tool uses a sophisticated blend of techniques to effectively locate miRNA binding sites and their corresponding heteroduplexes[18]. Using the online web server, the Fasta sequences of *Pyricularia oryzae* and selected mature miRNAs were incorporated into the tool. Target sites of the rice miRNAs were identified with the user-defined settings with Expectation = 8, seed region 2-7nt, HPS size 19, gap open penalty = 2, and Gap extending penalty = 0.5 (https://www.zhaolab.org/psRNATarget/).

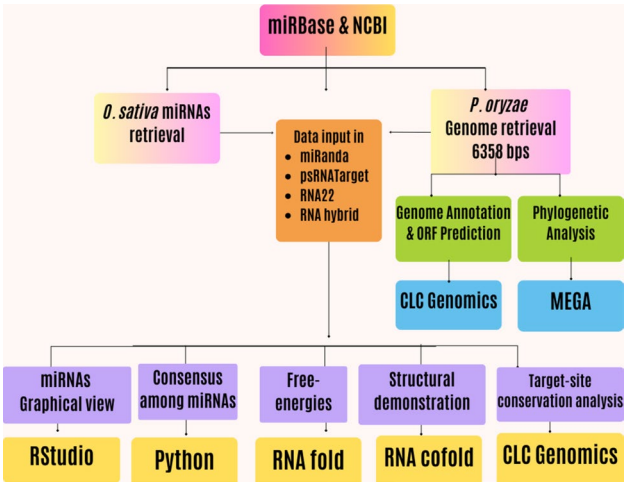


Fig. 1. The Schematic diagram shows the flowchart describing the designed research work.

Tools	Parameters	Source
psRNATarget	(Expectation = 8, penalty for opening gap = 2, penalty for extending gap = 0.5, penalty for G.U pair = 1, seed region = 2–7 nt and HPS size = 1	https://www.zhaolab.org/psRNATarget/
RNA22	Maximum folding energy = − 12 kcal/mol, Minimum number of paired-up bases = 12	https://cm.jefferson.edu/rna22/Interactive/
miRanda	Alignment Score Threshold = 140, Energy Threshold = − 20 kcal/mol	http://www.microrna.org/microrna/getDownloads.do
RNA hybrid	Energy Threshold = − 20 kcal/mol	https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid

Table 1. The parameters opted for computational miRNA target prediction algorithms in the present study.

Target prediction by RNA22

RNA22 is a web server and this approach is used because of its unique characteristics to research new miRNA-mRNA interactions[19]. A pattern-based approach is used by RNA22 to recognize miRNA binding sites and the heteroduplexes that bind to them. When a miRNA attaches to the target RNA sequence, a complex called a heteroduplex is created. The maximum folding energy for RNA22 was set to 12 kcal/mol when it was run under default conditions (https://cm.jefferson.edu/rna22/Interactive/).

Target prediction by miRanda

The miRanda is a popular technique that integrates crucial components of miRNA-target prediction, including the conservation level and miRNA 3′UTR site. To locate miRNA binding sites in the 3′ untranslated region (UTR) of target mRNAs, miRanda employs a dynamic programming approach[20]. The parameters opted for analysis were Score threshold 14.00, MFE threshold of − 20.00 kcal/mol, gap open penalty of − 9.00, and gap extending penalty − 4.00 (http://www.microrna.org/microrna/getDownloads.do).

Target prediction by RNAhybrid

The RNAhybrid miRNA target prediction tool, used for both long and short miRNAs, the RNAhybrid algorithm was utilized to calculate the minimum free energy[21]. The Fasta sequences of *Pyricularia oryzae* genome and selected miRNAs sequences were incorporated in this tool for analysis, using mfe set at − 20.00 kcal/mol (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid).

Secondary structure prediction by RNAfold and RNA cofold algorithm

RNAfold web server is a publicly available server that predicts the secondary structure of single-stranded miRNA precursors with a high degree of accuracy, by uploading the heteroduplex sequences on the webserver. RNAcofold algorithm finds out the minimum free energy of the miRNA-mRNA duplexes. Using default settings each unique duplex pair was uploaded on the server for analysis (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAcofold.cgi).

Statistical and graphical analysis

In this study, graphical representations of tabulated data from miRNA target prediction were created by employing R programming. R offers a wide range of libraries and functions for creating various types of plots, including Venn diagrams. Readxl and ggplot2 were used in graph prediction[22].

Mapping network interactions between miRNA and target fungus sequences

By utilizing the cutting-edge CIRCOS algorithm and Circos package v0.69-9R-Language, we were able to accurately map the interactions between predicted miRNAs and target fungus sequences with the help of a Circos plot[23].

Site conservation analysis of miRNA- *P. oryzae* genome

Analyzing the site conservation through protein BLAST analysis, *Pyricularia oryzae* isolates' genome sequences were downloaded from NCBI and integrated into CLC Genomics Workbench. When aligning the sequences using the greatest precision alignment option, the gap open cost was set to 10.0 and the gap extension cost to 1.0. To look into the conservation of binding site locations, miRNA binding sites were located from the alignment.

Phylogenetic analysis of *Pyricularia oryzae*

To create a phylogenetic tree and analyze the evolutionary relationships between isolates, *Pyricularia oryzae* genomes underwent multiple sequence alignment (MSA) and were imported into the MEGA (Molecular Evolutionary Genetic Analysis) software[24]. The Neighbor-joining (with Bootstrap value 100) and maximum likelihood approaches were used to create a rooted phylogenetic tree from the aligned sequences.

Cloning of over-expressed miRNA gene construction

The specific gene sequences responsible for the production of mature miRNAs were synthesized and cloned in plant expression vectors. The miRNA synthesizing gene (bp) driven under polyubiquitin (Zm-Ubi) coupled with transgene enhancer regulatory elements (HYL1) was integrated into the plant expression vector, pCAMBIA1301 between kpnI and HindIII restriction sites by fermentas gene ligation kit (catalogue). The figure shows the gene construction map of the miRNA clone.

Transformation of highly activated synthetic miRNA gene in rice callus

The transformation of gene construction in rice was administered through callus formation. The callus in the rice variety was induced by employing sterilized rice seeds from the variety. The sterilization of rice seeds was executed through ethanol and bleach and rinsing was done thoroughly with autoclaved water. The rinsing was done time and again to ensure the complete removal of bleach from seed samples. The rice seeds were allowed to germinate on MS medium without hormones at dark conditions keeping a temperature of 25 °C for 3–4 days consecutively. The scutellum was cut from the germinated seeds and placed on callus induction medium (MS medium, 4.43 g/L supplemented with 2,4-D at 2–3 mg/L, and sucrose was added by 30 g/L solidified with agar) in sterile Petri dishes. The incubation of explants was implemented in the dark at 25 °C to induce callus formation which was typically observed within 2–3 weeks. Sub-culturing of the callus was also done every 2–3 weeks onto fresh MS callus medium to ensure optimum growth. The designed synthetic gene construct was obtained

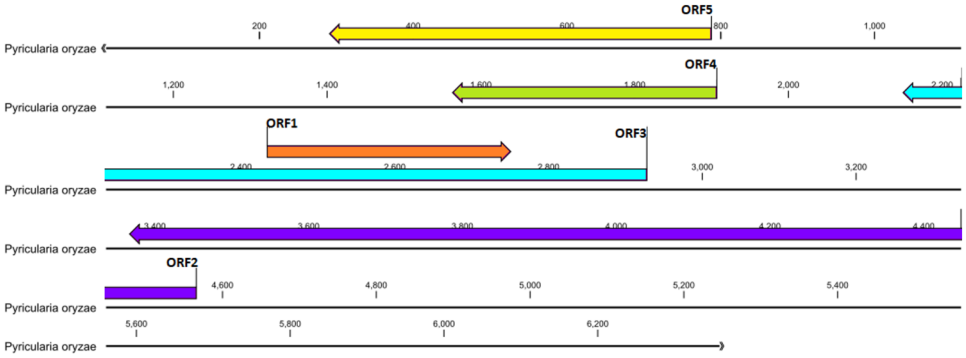


Fig. 2. The ORFs predicted in *Pyricularia oryzae* genome by. The genome (KY509032.1) has five ORFs distributed across it, each having different lengths, location and size.

No. of ORFs	Start	End	Length (nt|aa)	Strand	Start Codon
ORF1	2434	2751	318|105	Positive	ATG
ORF2	4566	3367	1200|399	Negative	ATG
ORF3	2928	2149	780|259	Negative	ATG
ORF4	1907	1563	345|114	Negative	ATG
ORF5	788	291	498|165	Negative	ATG

Table 2. Represents the number of ORFs, their location, starting codon, length, no. of their corresponding nucleotides, and translated amino acids of the *P. oryzae* genome.

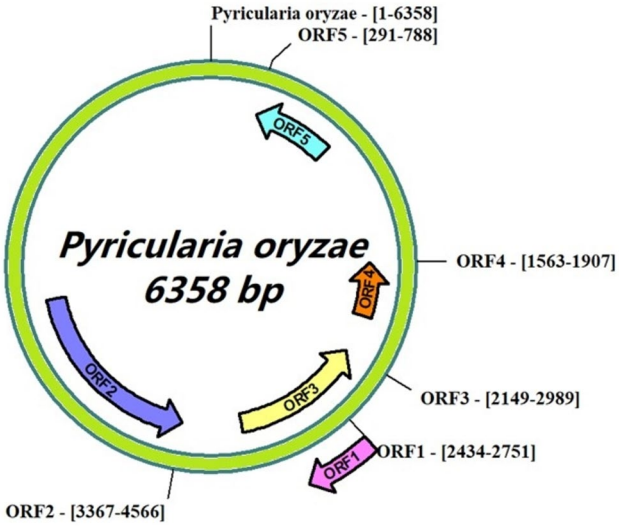


Fig. 3. Circular view of the *Pyricularia oryzae* genome (6358 bp) depicting its more active ORFs as shown in different arrows in various colours.

ORF Encoding protein

ORF1: 2434-2751

MHCVQRPHCLPFELFLPTTRDPCRIKVTSPPRQERHMYYVDTMVIIVHPVHHPEIDDPEKDGHIYTLDGL
QTCVNRRLVLKVFYKCPNRALADKGIAIVNQANFE

ORF2: 4566-3367

MHLRVMDELTSVEELRRQLREAEQRAEKEQQRAEREQQRAEEAEKERQQERQRAEASEQQTRPTTLDEYI
AACHDLVFSSFNIERDQKLTSKGPITNPRNKWCPTNVRPWSDFIQQQRITFGTVYDTFPADSRVFENRAF
LTGLGKRISVRKIADEKTLESFLHSSVEDPVKAIIGKLKEVEGVRSAFDLGNGIIFENHPHAISDVAEEV
VYRDTPSTPPATPNHSLDFNRLRPDQICVYRSNDAGSERRTMIYISEYKPPHKLTAPHLRVGLRPMNIYK
EVVNRKTIPTSMDPDARFQYHAERLTAAAITQTYHYIIEGGLEYGLLTTGEAIVFLKIDWREPETLFYHL
AEPNAEVLAHPDHFHLCTAVGQYLAFSLMALGLPGEKRLRGQDERHQAT

ORF3: 2928-2149

MALHNRQPSSHSNRNSGPSRACARHPINRAEFLNLLFEQLQRSLDKGITPLGYGGARGVLFKVCLVDYGY
TFVGKGTVRAFVKDLEHEAAVYTRLQPIQGVYVPVFLGVIDLRMMNRVYYYNHRVYVIHMTFLSWGGCDL
DATGITGGGEEELERKAMRSLHAVHQLGVAHKDVRGANMLFNAETSGVMMIDFERALLLEPPRPPLVQLA
SNKRKRKPDVVYPTKSGKPVGRSQVSRVFSEDIGLMKMAFDRPQPFQHE

ORF4: 1907-1563

MSRQAIAGLSTTIACKCFKQPPVCTAPGWVLQLSALQPSSGPFGGRLRLQSSSLRCSVVLGGGTGFCKSK
GPGLSKTSLWHRCAHPWTFGAFFSRKDDREQIQANSYDICDIYK

ORF5: 788-291

MVSASGGLLIGGGGLLLYEKCLDQFRKHPGLPRLYTAWGPNNGQDSVETAFMLGHSLPRFRSCPLPLPLF
PFLNISARTKTLEYCLPLPLYLLEYFVTVAHAFFQSLLILVRNLSHSPRLRRPQIRMNVIDKAVGGIVME
SIRAAPPDAIHRDQTRQHNLGEAGN

Fig. 4. The Amino acid sequences of the five predicted ORFs on the genome of *Pyricularia oryzae.*

in highly pure DNA concentration through maxi-prep to obtain 5 µg/ml concentration. For further applications, transfer callus to regeneration or transformation media as needed, ensuring aseptic conditions throughout the process.

PCR confirmation of miRNA genes in putative transgenic rice lines

The extraction of DNA from rice plants was done through 2% CTAB method. Fresh leaves from control and putative transgenic plants were collected, frozen in liquid nitrogen, and ground into a fine powder. The 2% CTAB extraction buffer was added and incubated at 65 °C for 30 min. The chloroform:isoamyl alcohol with the correct ratio (24:1) was added and centrifuged to separate different phases. The aqueous phase was transferred to a new tube and cold isopropanol was added. Samples were incubated at − 20 °C for 30 min to allow precipitation of

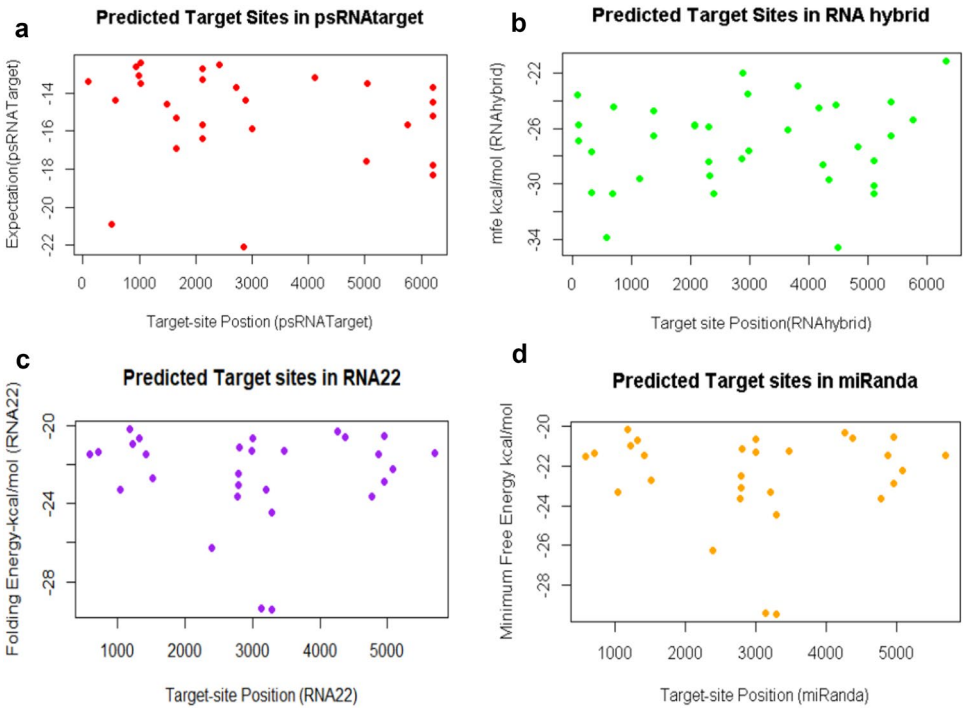


Fig. 5. Predicted target sites of *Oryza sativa* osa-miRNAs based on used four insilico miRNA targets. (**A**) Prediction of miRNA targets by psRNATarget (**B**) Prediction of miRNA targets by RNAhybrid (**C**) Prediction of miRNA targets by RNA22 (**D**) Prediction of miRNA targets by miRanda. The targets are represented by colored dots respectively.

Pyricularia oryzae ORFs	miRanda	RNA22	psRNATarget	RNAhybrid
ORF1 2434-2751	osa-miR166g-5p, osa-miR2926	osa-miR160a-5p, osa-miR160b-5p, osa-miR160c-5p, osa-miR160d-5p, osa-miR166b-5p	osa-miR169i, osa-miR171(a, b), osa-miR171d-5p	
ORF2 4566-3367	osa-miR 160c-3p, osa-miR164(a, b), osa-miR 166d-5p, osa-miR167(a, b), osa-miR395(h, j), osa-miR3696f-5p, osa-miR419,	osa-miR 160d-3p, osa-miR 160f-3p, osa-miR164(a, b)	osa-miR160a-3p, osa-miR 162(a, b), osa-miR 164(a, b, c), osa-miR166j-5p, osa-miR166m, osa-miR 393(a, b), osa-miR396a-5p	osa-miR156c-5p, osa-miR164a, osa-miR166c-5p, osa-miR167a-5p, osa-miR167b, osa-miR169a, osa-miR393a
ORF3 2928-2149	osa-miR 160d-3p, osa-miR 166g-5p, osa-miR 169f, osa-miR 393b-3p, osa-miR396e-3p, osa-miR 439(a, b, c, d), osa-miR528-3p, osa-miR530-5p, osa-miR815	osa-miR160a-5p, osa-miR166b-5p, osa-miR169(a, b), osa-miR395f	osa-miR164(a, b), osa-miR171a, osa-miR395w	osa-miR171a, osa-miR-394, osa-miR156h-3p, osa-miR396b-5p
ORF4 1907-1563	osa-miR414, osa-miR529b, osa-miR531(a, c)	osa-miR166b-5p, osa-miR166d-5p, osa-miR166h-5p	osa-miR169(a, c, e), osa-miR395u, osa-miR397a	
ORF5 788-291	osa-miR156f-3p, osa-miR156j-3p, osa-miR167c-3p, osa-miR395, osa-miR418	osa-miR160b-5p, osa-miR160c-5p, osa-miR160e-5p, osa-miR171a, osa-miR171e-5p	osa-miR162b, osa-miR166e-3p, osa-miR167e-3p, osa-miR171c-5p, osa-miR395v, osa-miR397(a, b)	osa-miR160a-3p, osa-miR160a-5p, osa-miR160c-3p, osa-miR160b-5p, osa-miR160c-5p, osa-miR160d-5p, osa-miR162b, osa-miR395c

Table 3. *Oryza sativa* predicted osa-miRNAs by four algorithms to target the five predicted ORFs of *Pyricularia oryzae* genome.

the rice DNA. The DNA pellet was obtained after centrifugation and the pellet was washed with 70% ethanol and air-dried. Finally, the DNA pellet was dissolved in TE buffer and the DNA quality was assessed by collecting concentrations. The putative rice transgenic plants with synthetic miRNA encoding gene construct were screened out with the help of primer specific PCR analysis. The primer sequences with forward, 3′-GCAGCA CGTTTCTTTCTTGC-5′ and reverse primer GCGTTGGGAGCTTGTACTTC were utilized to amplify transgene in putative transgenic rice plants.

RT-PCR of control and transgenic rice lines

The RNA extraction from control and transgenic rice leaves was executed by TRIZOL method. The Real Time PCR of control and infected transgenic rice plants was done to show mRNA expression of *Pyricularia oryzae* and control plants. The primer sequence used in Real Time PCR was 5′-ACGAAACGGGATTATGCAAG-3′.

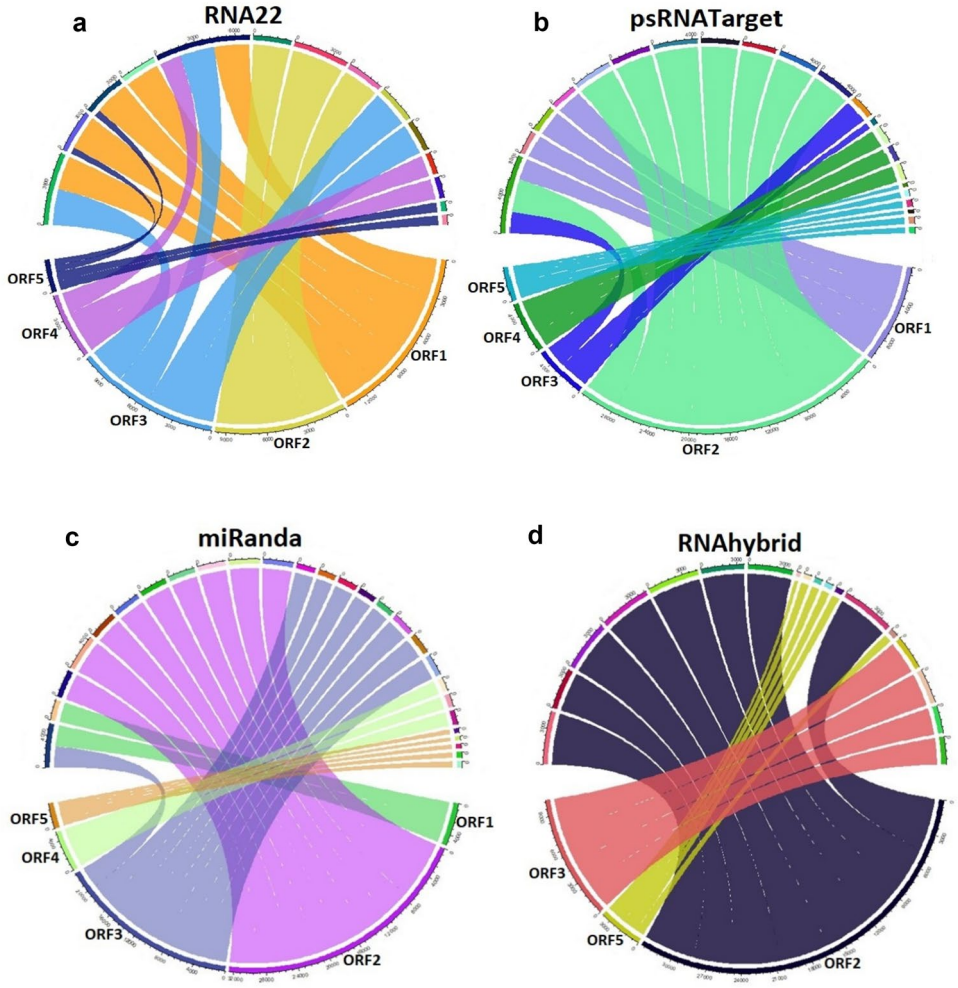


Fig. 6. The Circos plots showing rice encoded miRNAs targeting the *Pyricularia oryzae* ORFs. The above four Circos map shows the interaction between miRNAs targeting the five ORF for (**a**) RNA22, (**b**) psRNATarget, (**c**) miRanda, (**d**) RNAhybrid algorithms.

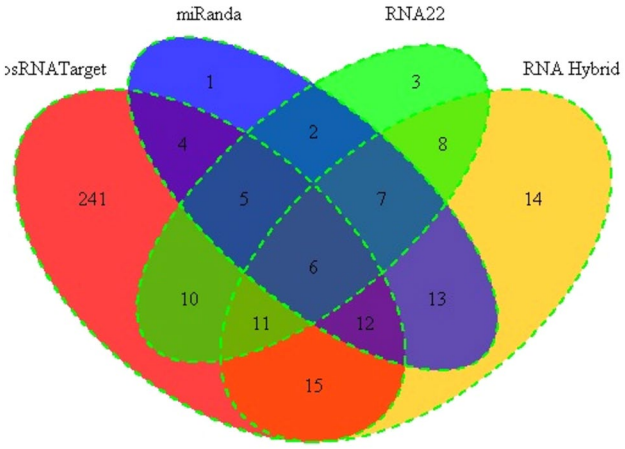


Fig. 7. Venn diagram that displays the precise targeting of *Pyricularia oryzae* genome by miRNAs derived from *Oryza sativa*. The Venn diagram reveals the total number of *Oryza sativa* encoded miRNAs predicted by miRanda, psRNATarget, RNA22, and RNAhybrid. It shows that six miRNAs were common in all four algorithms, 35 were common in at least three algorithm & 52 miRNAs were common in two computational algorithms. The diagram emphasizes the rigorous selection criteria for miRNAs, where only those predicted by a consensus of four diverse algorithms were chosen. These miRNAs exhibit effective inactivation of the *Pyricularia oryzae* genome.

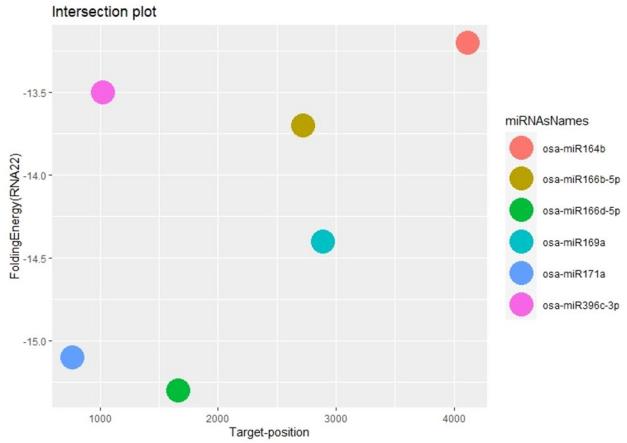


Fig. 8. Depiction of an Intersection plot, that gives a comprehensive overview of miRNA predicted, utilizing various bioinformatics algorithms. A color-coded key is provided alongside the plot for easy reference. Out of all the miRNAs predicted by four distinct computational algorithms, six miRNAs were identified as common to these computational algorithms. With the consensus of target prediction algorithms, the precise locations of the six targeted areas of *Oryza sativa* have been confidently predicted.

Oryza sativa miRNAs	Target-site miRanda	Target-site RNA22	Target-site psRNATarget	Target-site RNAhybrid	MFE miRanda	MFE RNA22	Expectation psRNATarget	MFE RNAhybrid
osa-miR164b	4427	4115	4489	4488	− 15.97	− 13.2	8	− 34.6
osa-miR166b-5p	3467	2719	5979	1135	− 21.27	− 13.7	8	− 29.6
osa-miR166d-5p	2117	1660	5177	5377	− 17.21	− 15.3	8	− 26.5
osa-miR169a	5979	2887	1877	3641	− 14.51	− 14.4	8	− 26.1
osa-miR171a	5291	762	2316	2865	− 20.43	− 15.4	7.5	− 28.2
osa-miR 396c-3p	1057	1022	2212	113	− 14.61	− 13.5	7.5	− 26.9

Table 4. Target binding sites of predicted consensus osa-miRNAs identified in the *P. oryzae* genome by four algorithms.

The mRNA expression of control as well as transgenic plants was calculated as Ct values which were then processed through the Relative Expression Software Tool (REST) to originate quantifications of mRNA expression in different rice lines. The evaluations and expression analysis indicate mRNA expression of *Pyricularia oryzae* in various rice lines control as well as transgenic lines.

Ethics approval

Not applicable. All authors have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of authors" as found in the Instructions for Authors and are aware that with minor exceptions, no changes can be made to authorship once the paper is submitted.

Ethical statement

It has been confirmed that the experimental data collection complied with relevant institutional, national, and international guidelines and legislation with appropriate permissions from authorities of the Department of Biotechnology, Knowledge Unit of Science, University of Management and Technology, Sialkot Campus, Punjab Pakistan.

Results

Sequence analysis and ORF of *Pyricularia oryzae*

The genome sequencing & ORF prediction of *Pyricularia oryzae* is depicted in Fig. 2 and Table 2, encompassing the sequences of nucleotides, and Open Reading Frames (ORFs). This information is crucial for comprehending the genetic makeup of this pathogenic fungus and its interactions with host plants (Fig. 3). Five ORFs were predicted in the genome of *P. oryzae*. ORFs are open reading frames within a DNA sequence, potentially code for proteins. Targeting the ORF can prevent the virus or pathogen from producing the proteins that it needs to survive and replicate (Fig. 4). Thus, it can lead to the death of the pathogen, or it can prevent the pathogen from causing disease.

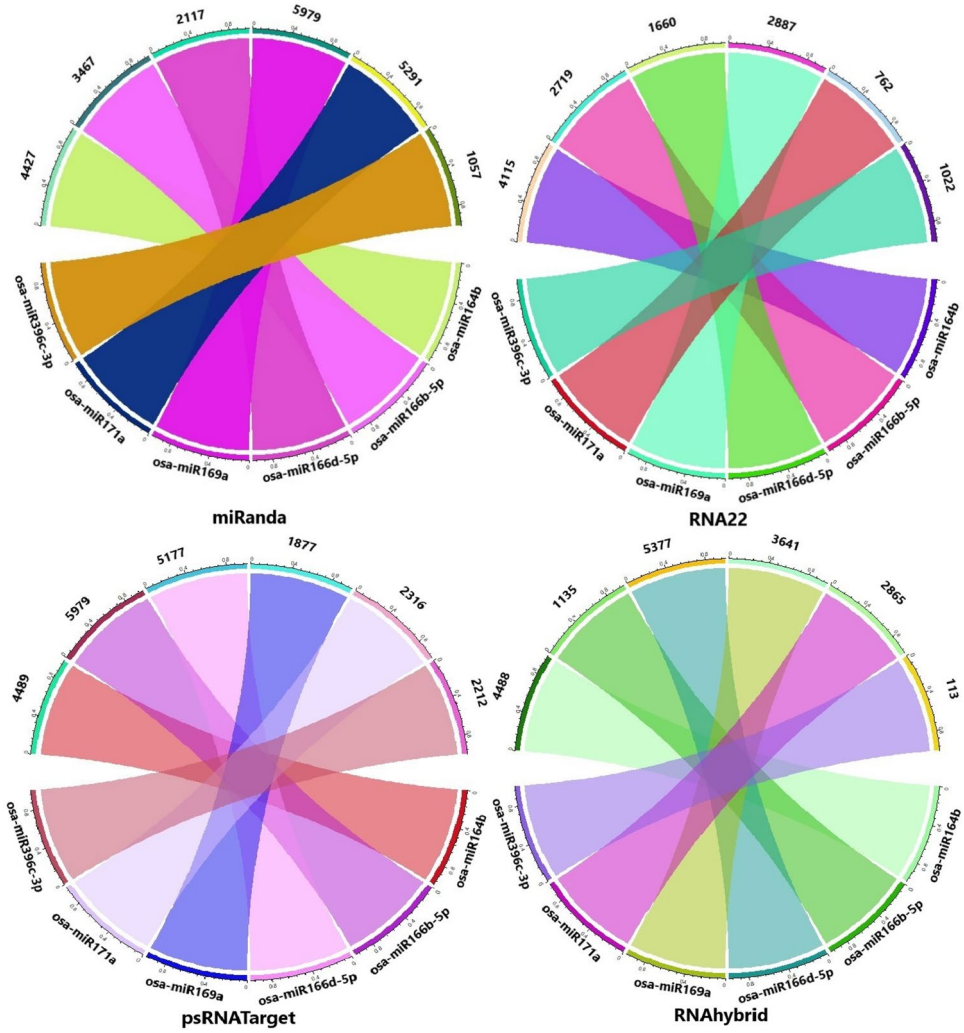


Fig. 9. Graphs showing the six consensus miRNAs that were common in each tool (i.e. miRanda, RNA22, psRNATarget, and RNAhybrid) with their respective target-site.

miRNA ID	Accession ID	Mature sequence (5′-3′)	Target binding locus positions	Target ORFs
osa-miR164b	MIMAT0000634	UGGAGAAGCAGGGCACGUGCA	4427, 4115, 4489, 4488	ORF2
osa-miR166b-5p	MIMAT0022856	GGAAUGUUGUCUGGCUCGGGG	3467, 2719, 5979, 1135	ORF1/ORF2/ORF3
osa-miR166d-5p	MIMAT0022858	GGAAUGUUGUCUGGCUCGAGG	2117, 1660, 5177, 5377	ORF4
osa-miR169a	MIMAT0000644	CAGCCAAGGAUGACUUGCCGA	5979, 2887, 1877, 3641	ORF3/ORF4
osa-miR171a	MIMAT0000645	UGAUUGAGCCGCGCCAAUAUC	5291, 762, 2316, 2865	ORF3/ORF5
osa-miR396c-3p	MIMAT0022865	GGUCAAGAAAGCUGUGGGAAG	1057, 1022, 2213, 113	ORF3

Table 5. Binding sites of predicted consensus *Oryza sativa* osa-miRNAs targets.

Target site predicted in four computational algorithms

Following graphs were generated by statistical data analysis using R (Fig. 5). Graphs show the predicted target sites in four different algorithms. The figures below represent the predicted target sites of *Oryza sativa* miRNAs in the *Pyricularia oryzae* genome by four algorithms (psRNATarget, RNA22, miRanda, and RNA hybrid) (Table 3). The colored dots show their respective target sites in the different regions in the genome of *P. oryzae* (Fig. 6).

Graphical representation of consensus of predicted miRNAs

The tabular data generated by four distinct target prediction algorithms underwent a rigorous transformation process utilizing the RStudio package. The data was then skillfully translated into visually appealing graphical

Oryza sativa miRNA ID	miRNA–mRNA target pair (heteroduplex)	ΔG duplex (Kcal/mol)	ΔG binding (Kcal/mol)
osa-miR164b	CGCAC-TGAGATCCTTTTCCC |||| || |||:||| ACGTGCACGGGACGAAGAGGT	− 9.00	− 7.42
osa-miR166b-5p	AAATCAGCTGGTAGCAATATTCT ||| | :|||:||||: GGGGCTCGGTC—TGTTGTAAGG	− 10.20	− 8.34
osa-miR166d-5p	CAACGATGCCAGAGAGAGGTTTT ||| |||||| :| :||:: GGAGCT-CGGTCT-GTTGTAAGG	− 11.00	− 9.17
osa-miR169a	GCGGTTGCTGTGAGAGCTGGGCTG |||: || | || ||||| AGCCG--TTCAGT-AGGAACCGAC	− 19.00	− 11.56
osa-miR171a	TATGT-AGCTTGGTTCAATTT ||:| || :||:|||||: CTATAACCGCGCCGAGTTAGT	− 11.30	− 9.73
osa-miR396c-3p	TCAGCTTCAGCTGCAGCTTGATC |: ||||| |||||: GAAGGGTGTCGA--AAGAACTGG	− 8.90	− 4.43

Table 6. Free energy (ΔG) estimates of the consensus of predicted osa-miRNAs. The ΔG of miRNA-mRNA interaction can be interpreted as follows: A negative ΔG indicates that the interaction is spontaneous and favorable. A positive ΔG indicates that the interaction is non-spontaneous and unfavorable.

representations using the readxl and ggplot2 functions, with the inclusion of a Venn Diagram to further enhance the presentation of results (Fig. 7).

According to results of four target prediction algorithms, six miRNAs can be confidently predicted: osa-miR171a, osa-miR166d-5p, osa-miR166b-5p, osa-miR164b, osa-miR396c-3p, osa-miR169a (Fig. 8). These miRNAs were common in four algorithms, target binding site of each miRNA predicted in four tools is mentioned in Table 4. Apart from that 35 were such miRNAs that were common in at least three computational algorithms and 52 miRNAs were common in two algorithms.

Predicted miRNAs have the potential to deactivate the genome of *P. oryzae*. The ORFs identified in *P. oryzae* are subject to inactivation by predicted miRNAs (Fig. 9). Specifically, ORF1 is targeted by osa-miR166b-5p, ORF2 by osa-miR164b and osa-miR166b-5p, ORF3 by osa-miR166b-5p, osa-miR169a, and osa-miR171a, ORF4 by osa-miR166d-5p and osa-miR169a, and ORF5 by osa-miR171a as depicted in Table 5. As a result, these ORFs are unable to translate into the resulting protein, ultimately leading to the silencing of the *P. oryzae* genome.

Free energies (ΔG) associated with optimal miRNA-mRNA interaction

The predicted six miRNAs by consensus of four algorithms were further evaluated by free energies. Free-energy estimation was obtained by RNAcofold web server of Vienna Package and shown in the following Table 6.

Secondary structure of predicted miRNAs

The secondary structures of miRNA precursors were obtained with utmost accuracy and precision using the RNAfold web server. Predicting the secondary structure of miRNA plays a crucial role in genome silencing. Table 7 shows the folding structure of six miRNA precursors osa-miR164b, osa-miR166b-5p, osa-miR166d-5p, osa-miR169a, osa-miR177a, osa-miR396c-3p.

Conservation analysis of predicted target sites in *P. oryzae* genome

Our study revealed that conserved miRNA target sites are significantly concentrated at the 3′ UTR and 5′ end of protein-coding genes, particularly in those genes associated with protein synthesis pathways. Furthermore, we discovered that the conserved nucleotides are present among various *P. oryzae* strains. As a result, we were able to enhance the precision and reliability of predicted miRNAs for these strains based on this conservation trend (Fig. 10).

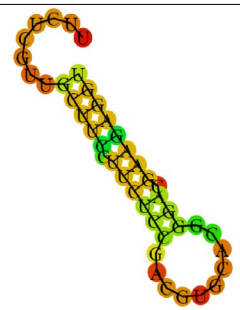
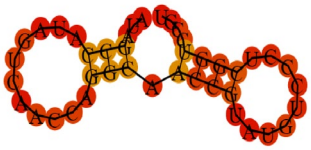
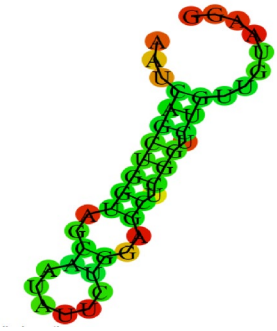
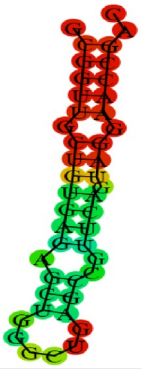
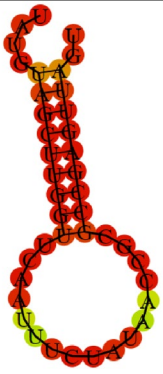
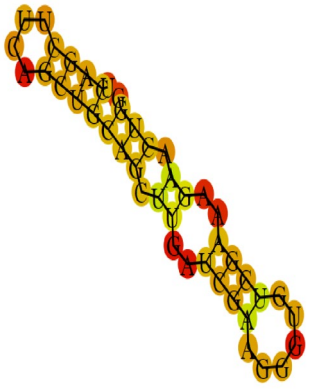
osa-miR164b CGCAC-TGAGATCCTTTTCCC |||| || |||:||| ACGTGCACGGGACGAAGAGGT	osa-miR166b-5p AAATCAGCTGGTAGCAATATTCT ||| | :|||:||||: GGGGCTCGGTC—TGTTGTAAGG
	
osa-miR166d- 5p CAACGATGCCAGAGAGAGGTTTT ||| |||||| :| :||:: GGAGCT-CGGTCT-GTTGTAAGG	osa-miR169a GCGGTTGCTGTGAGAGCTGGGCTG |||: || | ||||| AGCCG--TTCAGT-AGGAACCGAC
	
osa-miR171a TATGT-AGCTTGGTTCAATTT ||:| || :||:|||||: CTATAACCGCGCCGAGTTAGT	osa-miR396c-3p TCAGCTTCAGCTGCAGCTTGATC |: ||||| |||||: GAAGGGTGTCGA--AAGAACTGG
	

Table 7. Secondary structures of predicted *Oryza sativa* precursor miRNAs.

Phylogenetic analysis of *Pyricularia oryzae* strain

The phylogenetic tree of the genome of *Pyriculria oryzae* was constructed by using multiple sequence alignments of the isolated strain of the species (Figs. 11 and 12). To produce the most accurate phylogenetic trees, we utilized two robust methods: Maximum Likelihood and Neighbor-Joining (with Bootstrap value 100).

Results of trees show that, the closer the two species are on the tree, the more closely related they are. The length of the branches on the tree shows the amount of evolutionary time that has passed since the two species diverged. The nodes on the tree represent common ancestors. The nodes are points where two or more lineages

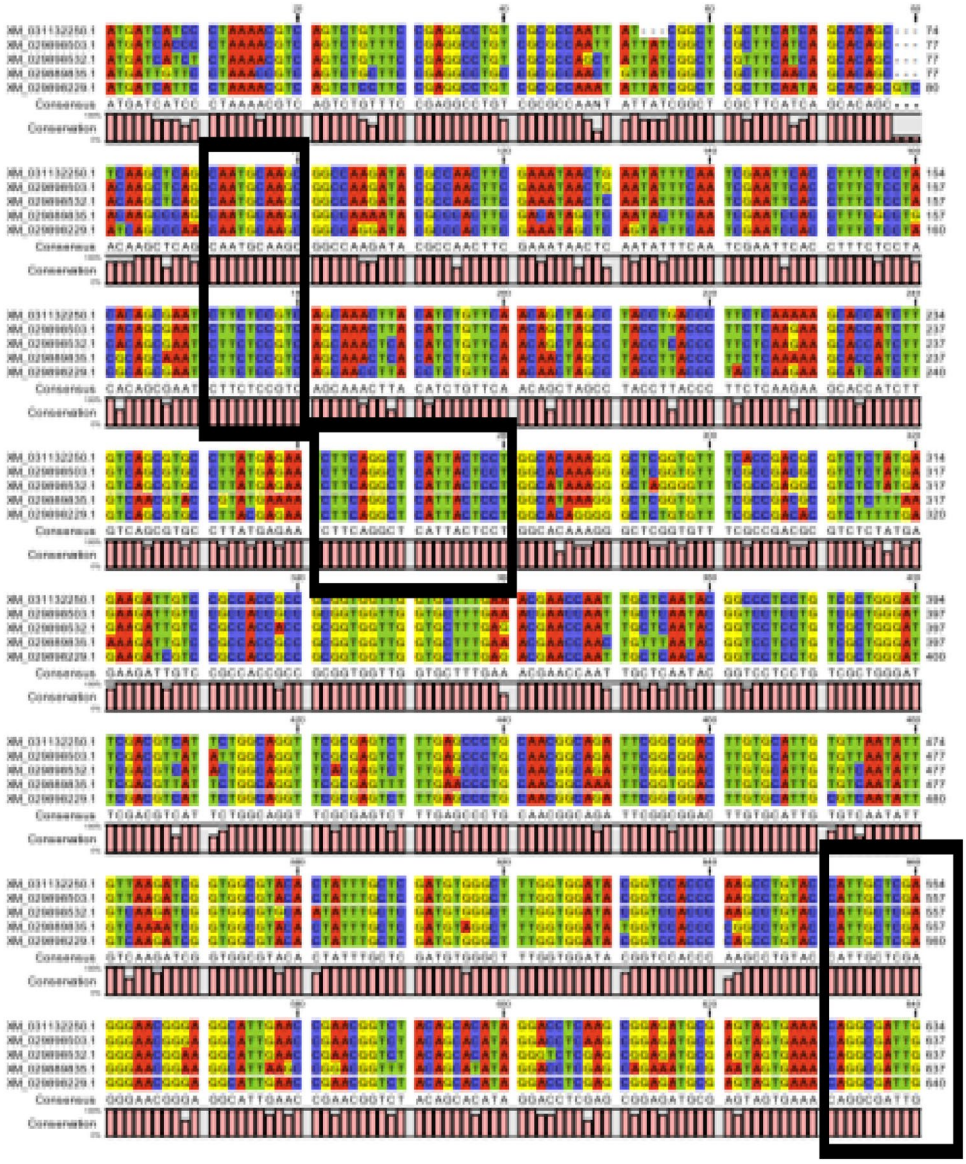


Fig. 10. Analysis of miRNA- *P. oryzae* genome binding site conservation. Four colors (red, yellow, green, blue) indicate the four nitrogenous bases. Red indicates Adenine, Yellow indicates Guanine, and Green & Blue indicate Thymine & Cytosine. The red bars in the above alignment show the percentage of conservation of the binding site from 0 to 100. The degree of conservation of the binding site can then be assessed by calculating the percentage of residues that are identical or similar between the aligned sequences. Marked sequences in the above figures show 100% conservation. Regions of the binding site that are highly conserved are more likely to be important for binding to the predicted miRNAs and are therefore more likely to be functional and considered potential target sites for predicted miRNAs.

diverge from each other. The more recent a node is, the more recent the common ancestor that it represents. The root is the point where all of the lineages on the tree converge.

Successful ligation and PCR confirmation

The successful ligation of transgene construct in plant expression vector was shown in 13A while PCR amilification of correponding gene was also represented in Fig. 13B

Experimental validation of potential miRNAs through real time PCR analysis

The Real Time analysis was performed in both control and transgenic rice lines which were further shown in Fig. 14. The mRNA expression level in control rice plants was higher than over-expressed miRNA transgenic lines. The rice lines in control plants indicated that mRNA expression remained high as the corresponding miRNAs could not bind efficiently with fungal mRNA while hybridization of miRNA with mRNA in transgenic lines lead to degradation of corresponding transgenic miRNA lines. The obtained expressed data in Fig. 14 is the

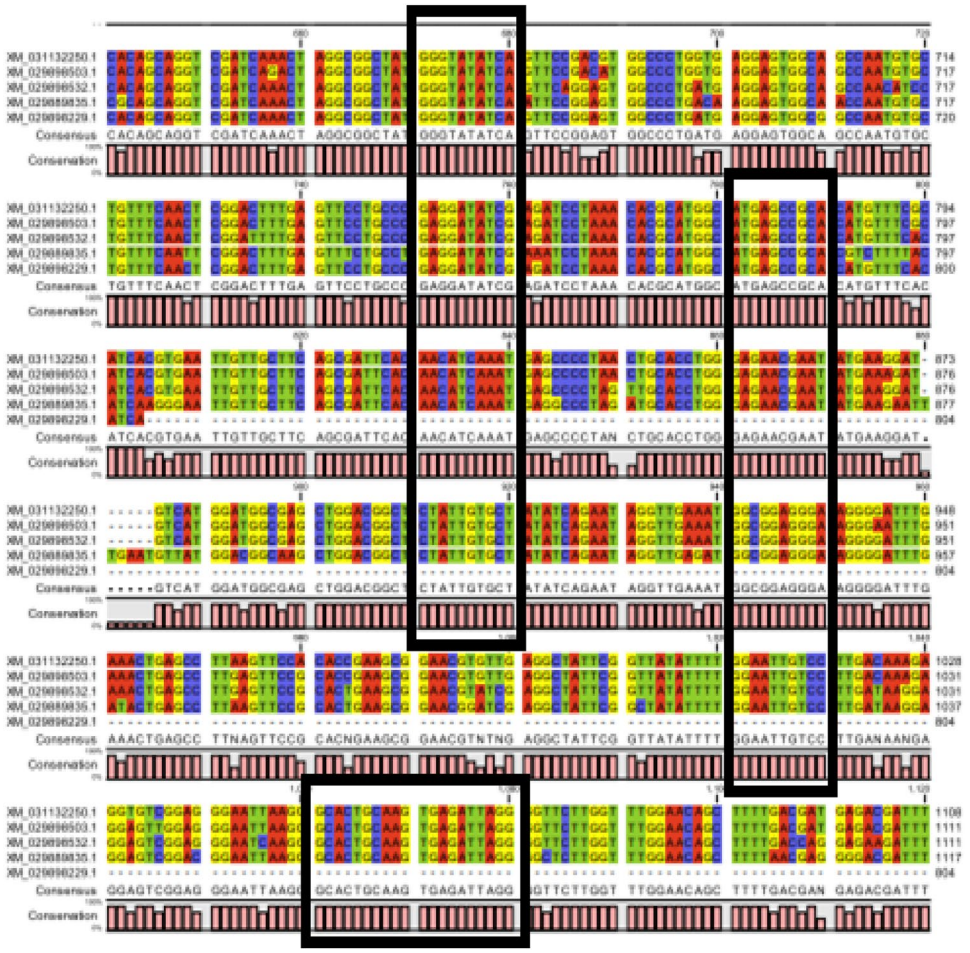


Fig. 10. (continued)

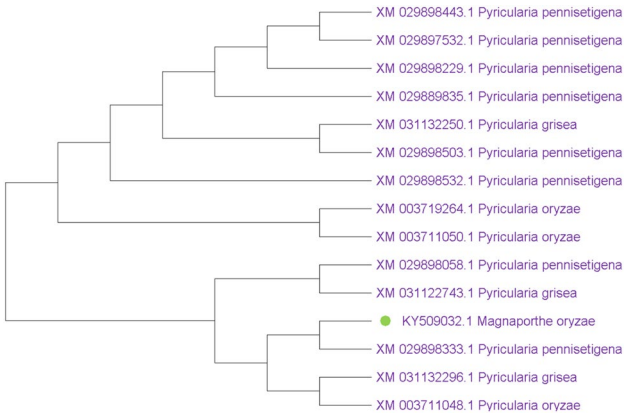


Fig. 11. Phylogenetic analysis of *P.oryzae* taxa by Maximum Likelihood method. The branches of a phylogenetic tree represent the evolutionary lineages that lead to different organisms. The green dot in the figure shows the strain under study. The closer two organisms are on a phylogenetic tree, the more recently they shared a common ancestor.

clear manifestation that those rice lines with enhanced biogenesis of mature miRNAs were successful in degradation of *Pyricularia oryzae* genome while control non transgenic lines showed increased level of fungus with high expression levels of mRNA. The trendline in the graph as indicated by dotted line represented the average mRNA expression of *Pyricularia oryzae* in enhanced miRNA rice lines.

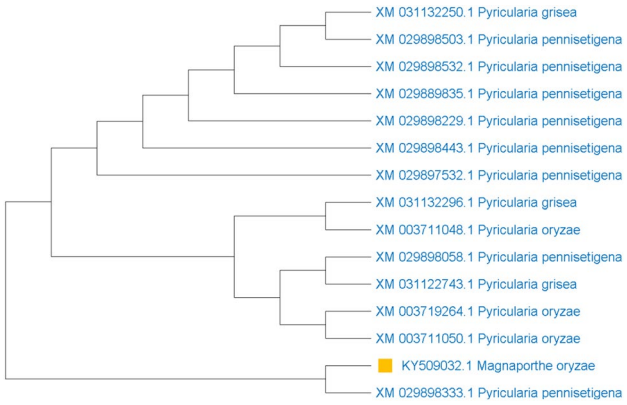


Fig. 12. Phylogenetic analysis of *P. oryzae* taxa by Neighbor-Joining method. The bootstrap value for Neighbor-joining was 100. The bootstrap values within a phylogenetic tree serve as an indicator of the frequency, out of 100, that the same branch is seen when regenerating the phylogenetic tree on a resampled data set. If we observe this occurrence 100 out of 100 times, it corroborates our findings. The branches of a phylogenetic tree represent the evolutionary lineages that lead to different organisms. The orange dot in the figure shows the strain under study. The closer two organisms are on a phylogenetic tree, the more recently they shared a common ancestor.

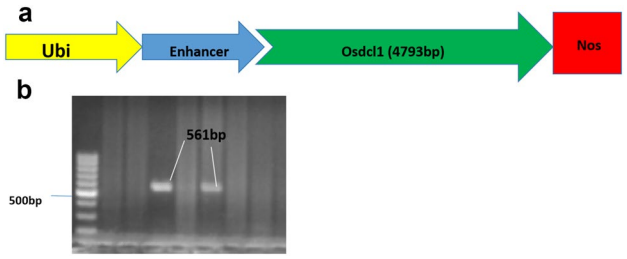


Fig. 13. The schematic diagram of cloning strategy used for miRNA synthesizing genes in Rice (**B**). The PCR amplifications of Osdcl1 (gene) producing 561 bp gene product confirming gene transformation in rice.

Discussion

Rice blast, a disease caused by a destructive fungus named *Pyricularia oryzae*, causes tremendous yearly loss to rice crops. The results of this study have successfully identified six miRNAs that have the potential to disrupt and inactivate the genome of *P. oryzae*. The discovery of these six miRNAs was made possible by utilizing four well-known computational algorithms. From the miRNAs predicted from these four tools, only those candidates were short-listed that were common in all four algorithms by using the intersection method. The six short-listed candidates were osa-miR171a, osa-miR166d-, osa-miR166b-5p, osa-miR164b, osa-miR396c-3p, osa-miR169a. These miRNAs were further evaluated by calculating their free energies, secondary structure prediction, and target-site conservation analysis. Moreover, phylogenetic trees were made to study the evolutionary relationship between the closely related isolates.

The results of this study have predicted six mature rice-encoded miRNAs that were expected to target the genome of *P. oryzae* towards developing the Rice blast disease in rice crops. The miRNAs identified in this study were predicted to interact with ORFs on the *P. oryzae* genome. Studies on miRNA target prediction for genome silencing have been extensive. Previous studies have reported the utilization of miRNA target prediction tools for silencing the genome of different pathogens[25], examples include RYMV[26], RTBV, RTSV[27,28], CMD[29], CLCuV[30], MCMV[31], ZYMV[32]. These studies involve computational approaches leveraging sequence complementarity, thermodynamic stability, and evolutionary conservation. No previous study was conducted on *P. oryzae* genome inactivation by using miRNA target prediction until now. However, there is a study published on the use of CRISPR/Cas 9 targeted knock-out of Rice susceptible genes. The results of this study supported that utilizing CRISPR/Cas9 to induce targeted mutations in rice susceptibility genes is a highly effective and innovative approach for developing resistance against Rice blast disease[33].

In this study four insilico algorithms were utilized, i.e., psRNATarget[18], RNA22[19], miRanda[20], and RNAhybrid[21] with default parameters to establish an effective computational method for the validation of miRNA target prediction results. The findings indicate that the five ORFs predicted in the *P. oryzae* genome are highly susceptible to targeting by consensus miRNAs. Among the six miRNAs predicted by consensus of four algorithms, silencing of the *P. oryzae* genome is the direct result of the targeting of ORF1 by osa-miR166b-5p, ORF2 by osa-miR164b and osa-miR166b-5p, ORF3 by osa-miR166b-5p, osa-miR169a, and osa-miR171a, ORF4 by osa-miR166d-5p and

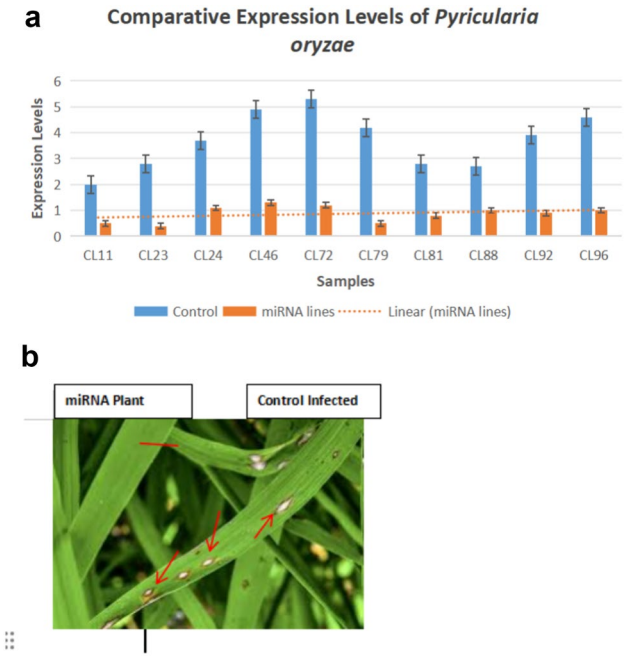


Fig. 14. The graphical demonstration of *Pyricularia oryzae* based mRNA expression in control and transgenic rice lines. The blue bars in the graph inidcated higher mRNA expression in lines while orange bars showed very lower mRNA expression in transgenic lines. (**B**) The (**B**) indicates and showed the difference between control and transgenic miRNA line which triggered gene silencing in rice against *Pyricularia oryzae.*

osa-miR169a, and ORF5 by osa-miR171a. In other words, the inability of these ORFs to translate into resulting protein is a direct consequence of the aforementioned targeting, and there is no room for doubt in this regard.

Our study confirmed the previous findings emphasizing the crucial role of miRNAs in plant defense against pathogens. For example, research has demonstrated differential expression of miRNAs in rice in response to *P. oryzae* infection, with some of these miRNAs regulating defense-related genes[34]. Our study significantly advances previous findings by pinpointing specific miRNAs capable of targeting the *P. oryzae* genome, potentially boosting rice resistance. The study has identified six candidate miRNAs belonging to different miRNA families, each with unique functions in plant development and defense. For instance, the miR166 family is known to regulate class III homeodomain-leucine zipper genes, playing a significant role in plant development and stress responses[34]. In a similar vein, the miR164 family is known to target NAC transcription factors, which are crucial for plant defense against pathogens[35]. Based on our study, the presence of these miRNAs strongly indicates their influential role in regulating defense-related genes in rice, potentially leading to enhanced resistance against *P. oryzae.*

The free energy calculations and secondary structure analysis of these miRNAs conclusively demonstrate their potential to form stable complexes with their target sites. Calculating free energy is a crucial factor in determining the stability and efficacy of miRNA-mediated gene silencing. Our findings unequivocally demonstrate that all six miRNAs exhibit highly favorable free energy values, signifying robust interactions with their corresponding target sites in the *P. oryzae* genome. The consistently low free energy values strongly support the formation of stable complexes between these miRNAs and their target mRNAs, ultimately resulting in potent gene silencing. Furthermore, the target site conservation analysis strongly supports the idea that these miRNAs are conserved across different plant species, including rice, and have evolved to target specific genes involved in defense responses. In addition, the phylogenetic trees constructed using the Maximum Likelihood and NJ methods indicate that the closest relative is XM029898333.1, which is identified as the sister taxa of the strain KY509032.1.

Genome silencing by miRNA target prediction is a promising new approach to controlling rice blast disease. This approach can potentially be more effective and less harmful to the environment than other methods of controlling the disease[36–38]. The implementation of biological control using microbial agents with antimicrobial properties presents a remarkable alternative for managing Rice blast, however, it can be challenging to achieve in the field as its effectiveness is highly dependent on weather conditions[39]. The advantage of using genome silencing by miRNA target prediction to control rice blast disease is that it is a highly specific approach that can target specific genes involved in the pathogenesis of the disease[40]. Moreover, it is a relatively safe approach that does not involve the use of chemicals or other harmful substances[41]. Experimental validation remains crucial to confirm predicted targets due to the complexity of miRNA-target interactions and the need to minimize false positives.

Conclusion

This work confidently identified the potential candidate miRNAs that are most suitable for RNAi-mediated silencing of the *P. oryzae* genome. The identification is based on a consensus of plant miRNA target prediction methods. Further, experiments on wet laboratory provided strong evidence that these miRNAs were highly effective in

controlling symptoms of *Pyricularia oryzae* in rice crop. Six miRNAs were predicted from the consensus of four algorithms: osa-miR171a, osa-miR166d-, osa-miR166b-5p, osa-miR164b, osa-miR396c-3p, osa-miR169a. These miRNAs were further evaluated by calculating their free energies, and secondary structures, and by thorough evaluation, it was revealed that the bond between miRNA-mRNA duplex is spontaneous and favorable. Site conservation analysis of *P. oryzae* isolates revealed the information about degree of conservation, and the higher percentage of conservation suggests the likelihood of potential target sites for predicted miRNAs. Moreover, phylogenetic analysis indicated the most closely related *P. oryzae* strains based on their evolutionary relationship.

Data availability

The complete genome of *Pyricularia oryzae* with size 6358bps was retrieved from NCBI (Accession no. KY509032.1) and copied in another separate FASTA file (https://www.ncbi.nlm.nih.gov/). The datasets used and/or analyzed during the current study available in the manuscript file.

Received: 8 March 2024; Accepted: 9 September 2024
Published online: 18 September 2024

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Acknowledgements

The authors are thankful to the Deanship of Scientific Research, King Khalid University, Abha, Saudi Arabia for Financially supporting this work through a large research Group Project under grant no. R.G.P. 2/369/44.

Author contributions

T.S. carried out research work and wrote the initial draft of the manuscript. M.F.A. and S.A. conducted the data analysis. M.F.S., Q.A., and S.I. planned and supervised the study and edited the final version of the manuscript. M.Y.A., M.A.J. and Q.A. technically reviewed and finalized the draft. All authors reviewed the final version of the manuscript and approved it for publication.

Funding

No funding was obtained for this study.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-72608-4.

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