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Identification and characterization of differentially expressed novel miRNAs (21–24 nt) in a *Macrophomina phaseolina* resistant RIL line of jute (*Corchorus capsularis* L.)

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ABSTRACT

A RIL population of jute developed by crossing one resistant accession CIM 036 and a susceptible variety JRC 412 was used to identify novel defence related miRNAs activated upon challenged inoculation with stem rot pathogen *Macrophomina phaseolina*. About a total of 15.7 million reads were generated from the resistant line with the read length of 1 × 50 bp. Here, we identified nine mature novel microRNAs which passed Minimum Free Energy (MFE Kcal/mol) criteria. Target site and secondary structure were predicted and most of them showed ubiquitination and selective autophagy activity with high expression value. Five novel miRNAs viz. Candidate_41, Candidate_9, Candidate_66, Candidate_65 and Candidate_8 had free energy less than −25 kcal/mol. Known microRNAs viz. miR-845b and miR-166 superfamily are abundantly expressed with high expression value. The sequence of jute miR-845b superfamily is identical to that of *Arabidopsis thaliana* except at 18th position, but unlike in *A. thaliana* it targets the coding sequence for the P-loop motif in the mRNA sequences for disease resistance proteins with nucleotide binding site (NBS) and leucine-rich repeat (LRR) motifs. *In-silico* analysis suggested that miR-845b and miR-166 superfamily provided NBS-LRR and ROS mediated defence and subsequently expression of novel microRNAs with selective autophagy activity enabled multi-layered defence cascade against *M. phaseolina* in jute.

1. Introduction

Jute is an important natural fibre which is extracted from the stem bark of jute plants (*Corchorus capsularis, Corchorus olitorius*). The crop is generally cultivated in South East Asian and few South American countries. The major jute producing countries are India, Bangladesh and China. Other countries viz., Uzbekistan, Thailand, Nepal, Myanmar, Brazil, Vietnam etc also produce jute. Jute fibre is mainly used in packaging, textiles and in making many diversified products. Jute plants are infected by a number of plant pathogens among which *Macrophomina phaseolina* is the most important one. The necrotrophic fungal pathogen mainly causes leaf blight and stem rot symptoms in jute. The disease causes about 10–40% crop loss depending on the severity of infection [1].

MicroRNAs (miRNAs) are versatile regulators of gene expression in plants and animals. They are 21–24 nucleotides long and processed by a Dicer nuclease from long RNA precursors with base paired foldback structures [2]. The single-stranded form of the miRNA forms a ribonucleoprotein complex with Argonaute (AGO) that can bind by base pairing to a target RNA [3,4]. In plants, the successful targeting reaction requires complementarity of the miRNA at most of the residues [5].

In some instances, miRNA-mediated gene silencing is a simple negative switch: whenever the miRNA gene is active the target mRNA is silent. However, these versatile RNA regulators may also participate in feedback loops and carry out more subtle roles in genetic regulation. They might dampen fluctuations in target gene expression, for example, influencing temporal changes [4]. The miRNAs or their precursors may move through plasmodesmata and different stages of the feedback system occur in adjacent cells or in separate roots and shoots [6,7].

miRNAs could also initiate regulatory cascades with multiple mRNA targets [8]. These cascades involve secondary small interfering RNAs (siRNAs) that associate with AGO proteins, similarly to miRNAs. The first step in these cascades requires an RNA-

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dependent RNA polymerase (RDR; RDR6 in *Arabidopsis thaliana*) and it takes place when the initiator miRNA duplex structure is asymmetrical [9] or when the initiator miRNA is of 22 nucleotides rather than 21-nucleotides long [10,11], or if there are two target sites for 21-nucleotide RNAs [12]. The initiator miRNA stimulates the RDR to convert the targeted RNA into long, double-stranded RNA that is then processed by Dicer into secondary siRNAs. A high proportion of the secondary siRNAs are in a 21-nucleotide phased in which the first position is the cleavage target of the initiator miRNA [13].

In the present study, we identified novel miRNAs and validated novel host pathogen interaction targets. By integrating PARE data with small RNA data and novel bioinformatics analyses, we identified 42 new miRNA candidates from 51 precursors in 21 resistant RIL lines. We observed, miR-845b and miR-166 superfamily are abundantly expressed with high expression value. miR-845b's family targets the coding sequence for the P-loop motif in the mRNA sequences for disease resistance proteins with nucleotide binding site (NBS) and leucine-rich repeat (LRR) motifs. miR-166 superfamily regulated ROS mediated (Reactive oxygen species) gene regulation. Here, we identified nine mature novel microRNAs and most of them showed ubiquitination and selective autophagy activity.

2. Material and methods

2.1. Plant material

A RIL (recombinant inbred line) population of jute was developed by crossing one resistant accession CIM 036 and a susceptible variety JRC 412 and it consisted of 177 lines [14]. A *M. phaseolina* resistant line no.75 and a variety JRC 212 as healthy control were used in present study.

2.1.1. Fungal culture maintenance and inoculation

The pathogen *M. phaseolina* was isolated from infected jute (*C. capsularis*) plant (cultivar JRC 412) at the research farm of Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India. The fungus culture was maintained at 25 °C on potato dextrose agar (PDA). 3 days old fungal culture was used for challenged inoculation. One week old jute plants were sprayed with fungal suspension containing 6.2×10^3 cfu per ml and the inoculum was prepared following the procedure described by Biswas et al., 2013 [15]. Untreated healthy plants served as control. Leaf samples were taken three days after inoculation and the tissues were used for mRNA isolation. Parent susceptible variety JRC 412 was taken as healthy control.

2.2. Total RNA isolation and its qualitative and quantitative analysis

Total RNA was isolated from the leaf samples of treated as well as untreated plants using Trizol (Invitrogen, NY, USA) as per manufacturer's instructions. The quality of total RNA was checked on 1% agarose gel loading 5 μl samples for the presence of 28S and 18S bands. The gel was run at 100 V for 30 min. Further, total RNA was quantified using Qubit fluorometer (Invitrogen, NY, USA). Sample RNA concentration was 73.6 ng/μl and the yield was 3.68 μg.

2.3. Small RNA library preparation

The small RNA library was prepared from 1 μg total RNA using Illumina Trueseq Small RNA kit. Initially, 3′ and 5′ adaptors were ligated to each end of the RNA molecule and RT reaction was used to create single stranded cDNA. This cDNA was then PCR amplified using a common primer and index primer to create double strand. The cDNA library was then purified using 6% Novex TBE PAGE gel. After gel purification, the cDNA was extracted and concentrated by ethanol precipitation. Final Library was validated on Bio-analyzer 2100 (Agilent technology, CA, USA) using High-Sensitive DNA Chip. The mean size of the fragment distribution was 159 bp. The library was sequenced using 1×50 bp chemistry. Library profile of small RNA covered average size of 159 bp and size distribution CV was 8.8 (Fig. S1).

2.4. Workflow of bioinformatics analysis of small RNA

To detect known and unique small RNAs and to predict their target, Illumina Trueseq data were imported from CLC workbench. Raw reads were used for mapping against Rfam. 20–26 bp long small RNA was extracted and trimmed. Using RNA analyzer trimmed reads were mapped to draft genome. Possible novel miRNAs and their precursors were generated. The precursors were used for generating secondary structure and novel miRNA prediction.

2.4.1. Genome information

Untreated healthy jute (*C. capsularis*) (cultivar JRC 212) was used as the reference genome for miRNAs identification as well as for the target prediction.

2.5. miRBase

The miRBase is a searchable online repository for published microRNA sequences and their associated annotations. The current release of miRBase (Version 21) was used to identify known microRNAs in the studied sample. miRNAs were identified from healthy as well as from inoculated plants. After identification of all the microRNAs, we indicated novel microRNAs from inoculated plants.

2.6. Trimming

The raw reads of studied samples were mapped against Rfam database (collection of non-coding RNA families) to remove the contamination, and then imported by discarding the read names and quality Phred score with CLC genomics workbench using the Illumina importer. Once the reads were imported, they were filtered for adapter sequences. While trimming, the maximum length of the reads was set to 24 bp.

2.7. Identification of novel miRNAs

MiR analyzer pipeline was used to predict potential miRNA precursor molecules in *M. phaseolina* infected jute. In the next step, secondary structures were predicted for precursors of potential candidate novel miRNAs by using m-Fold web server with default parameters. miRNAs whose precursor's secondary structure having free energy equal to or less than −21 kcal per mol and having the length between 20 and 24 bp were considered as novel miRNAs. This resulted in 15 novel miRNAs.

2.8. Target prediction of mature known and novel miRNAs

Targets were predicted for known and novel miRNAs of resistant line using web based tool psRNA (www.plantgrn.noble.org/psRNATarget/). The program uses a scale of 0–5 for the flexibility of miRNA-target pairing with the smaller numbers representing higher stringency, while score of 3 was used in this analysis. Healthy sample was used as reference for target searches. Following parameters were used for psRNA. Target program: Maximum expectation = 3.0, Length for complementarity scoring

(hspsize) = 20, Target accessibility allowed maximum energy to unpair the target site (UPE) = 25 or less, Range of central mismatch leading to translational inhibition = 9–11 nt.

2.9. Alignment

The small RNA high-throughput sequencing libraries were aligned to the reference sequence using the PatMaN [16] alignment program. Only reads with 100% match to the genome were used in further analysis.

2.10. Secondary structure prediction

Secondary structure of novel miRNAs were predicted using mFold server with default optimized parameters. Mature novel miRNA id was auto generated by miRanalyzer which passes the essential Minimum Free Energy (MFE) criteria.

2.11. Identifying miR-845b and miR-166 diversity, sequence alignments, and its targets

The UEA small RNA analysis toolkit [17] was used to identify members of a given miRNA family (miRProf and miRCat). Sequences of miR-845b and miR-166 members were obtained from miRBase release [18] and aligned using ClustalW and ClustalX2 [19]. Protein sequence logos were generated using seqLogos (http://imed.med.ucm.es/Tools/seqlogo.html). Targets of miRNA were identified using two different algorithms, namely, psRNA Target algorithm [20] and TAPIR algorithm [21]. To find targets of miR-845b and miR-166 family in the *Arabidopsis thaliana* genome, target NBS-LRR sequences and peroxidase regulatory sequence were taken from the NCBI. Images were generated using the UEA small RNA toolkit function SiLoMa [17]. Analysis of abundance of novel miRNA superfamily was performed through miRProf analysis of published large-scale data sets derived from various plant species available through the Gene Expression Omnibus (GEO) platform [22]. miRNA sequences were checked to compensate for the annotation of miR-845b type and miR-166-type and sequences in miRBase.

2.12. miRNA in situ hybridization

miRNA expression was assessed following the protocol of Silahtaroglu et al. (2007) [23]. In brief, after a 4 h pre-hybridization, a 50 FITC labelled miRCURY LNA probe targeting −microRNAs (Exiqon) were hybridized to proteinase K-treated 10 mm sections at 55 °C for 12 h. Slides were then incubated with anti-FITC-HRP (BioRad) and the resulting signal was intensified with the TSA Plus Fluorescein System (BioRad).

3. Results

3.1. Identification of known and novel microRNAs in resistant RIL line

Two steps strategy was used to identify known and novel miRNAs viz. step 1: The small RNAs were mapped to the miRBase Release 21 to identify known miRNAs and step 2: The small RNAs were mapped to the CDS of untreated healthy control of *C. capsularis* to identify novel miRNAs using miRanalyzer version 3 with default parameters (allow mismatch to genome: 1, score (minimum prediction score): 0.9, minimum read count: 1).

3.2. Expression of mature known miRNAs

To identify the known miRNAs, small RNAs were annotated against miRBase database by using CLC Workbench (version 6) and two maximum mismatches were allowed in the annotation process. 132 miRNAs were identified from the resistant line among which miR-166b/miR-166j/miR-166u is highly expressed with expression value 7915 (Additional File 1). The most abundant species is *Glycine* max followed by *Arabidopsis* sp. Other than miR-166; miR-156a, miR-4995 and miR-845b were found to have high expression values. miR-166b/miR-166j responsible for NADP dehydrogenase oxidoreductase mediated ROS response, whereas miR156a had pectinase activity, miR4995 activated thioredoxin 1 pathway, miR482a/miR1957a indicated NBS/LLR class defence and miR 482f directly showed peroxidase activity. miR 8109 with high expression value activated 26S ribosomal subunit of systemic defence response. miR156 activated the gene related to Chr8 scaffold −27. miR-6300, miR-8117, miR-5119, miR-156a/c, miR-6244 and miR-482c had medium expression value with various known systemic defence gene related activity viz. zinc finger DNA binding protein, transcript inhibitor response 1, SBP transcription factor, Teosinte glume architecture 1, Excinuclease ABC C subunit, LIM domain protein, MICAL C-terminal-like protein etc. Other small RNAs with low expression value was related to cellular or different metabolic processes like Mitochondrial-processing peptidase subunit alpha, Glutathione S-transferase GST 19, other chromosomal scaffold, Late embryogenesis abundant protein Lea5, Acetolactate synthase etc. (Table S1).

3.3. Target prediction of known miRNA

A total of 132 known miRNAs were searched for targets against healthy *C. capsularis* CDS region. Out of 132 known miRNAs, 63 miRNAs were found to have 661 targets in the *C. capsularis* (Table S2). miR-845b, miR-5658, miR-3613b, miR-4502, miR-2406, miR-2127, miR-156a, miR-156b, miR-7086, miR-156f, miR-4249, miR-5721, miR-159, miR-482c, miR-530a, miR-156g, miR-482a, miR-396e and miR-477f were maximum number of targets (Table S3). NADPH oxidoreductase and cell wall peroxidase gene played a major role in ROS mediated plant defence. ROS established SA mediated systemic defences, Systemic Acquired Resistance (SAR).

3.4. Expression and target prediction of mature unique miRNAs

MiRanalyzer pipeline was used to predict potential miRNA precursor molecules in resistant line by mapping them on CDS of healthy *C. capsularis*. The precursor molecules were extracted from CDS. Secondary structures were predicted for precursors of potential candidate novel miRNAs by using m-Fold web server with default parameters. miRNAs whose precursor's secondary structure having free energy equal or less than −21 kcal per mol and having the length between 20 and 24 bp were considered as novel miRNAs. This outcome suggested that we identified 15 novel microRNAs (Table S4). A total of 15 novel miRNAs were searched for targets against the CDS region of healthy *C. capsularis*. A total of 9 unique novel miRNAs (out of 15) were found to have 24 targets in the resistant line. The targets are shown in Table S5. Al the microRNAs target E3 ubiquitin ligase gene family and regulate degradation.

3.5. Predicted novel microRNAs secondary structure

miRNA named candidate _41, candidate _17, candidate _81, candidate _9, candidate _5, candidate _66, candidate _69, candidate _13, candidate _20, candidate _65, candidate _8, candidate _68,

candidate _15, candidate _19 and candidate _18 passed the MFE criteria and their precursor and secondary structure were listed in Table S6.

3.6. Validation of novel microRNA

The immunoblot assay showed that micro RNA namely candidate_81, candidate_9 and candidate _17 targeted PUB, CULLIN and Fbox gene respectively (Fig. 3).

3.7. An unusual diversity known miRNA-845b superfamily

A wide sequence variation was observed in miR-845 super family. Jute miR-845b family was identical with *Arabidopsis lyrata* miR-845b family, whereas miR-845b of *Arabidopsis thaliana* varied in 18th uracil base position (Fig. 1).

3.8. miR-166 family diversity

The plant miR-166 microRNA precursor is a small non-coding RNA gene. This microRNA (miRNA) has now been predicted or experimentally confirmed in a wide range of plant species. microRNAs are transcribed as ~70 nucleotide precursors and are subsequently processed by the Dicer enzyme to give a ~22 nucleotide. There are some examples of distantly related plant species with an identical isoform of miR-166. miRNAs showed distinct similarity among jute Cc-miR-166a, pab-miRNA166a (*Picea abies)* and mtr-miR-166c (*Medicago truncatula*); mes-miR-166j (*Manihot esculanta*) and aqc-miR-166d (*Aquilegia caerulea*). *Arabidopsis thaliana* miR-166 gene family displayed wide diversity among 2nd, 4th, 5th, 7th and 12th position. *Glycine* max and *Phycomotrella patens* have single nucleotide variation in 4th and 11th position respectively (Fig. 2).

4. Discussion

MicroRNAs (miRNAs) are endogenous non-coding RNA molecules (see Fig. 3). These miRNAs can regulate gene expression post-transcriptionally by binding to the 3′-untranslated region (3′-UTR) of target genes to promote mRNA degradation or protein translation inhibition [24]. Thus, they play important roles in various biological processes, such as embryo development, cell proliferation and differentiation, and defences [25,26].

In the present study we identified nine novel microRNAs and all of them target Ubiquitin ligase gene family. The ubiquitination cascade is one of the main pathways of post-translational regulation of gene expression in eukaryotic cells, in which ubiquitin is bound to a lysine residue of a target protein. Being a reversible form of covalent modification, ubiquitination acts very rapidly on target protein in different ways. The best characterised function of ubiquitination is the degradation of target proteins through the 26S proteasome.

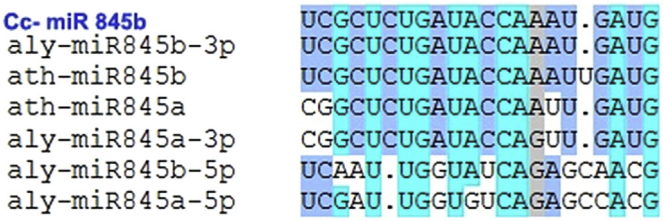

Fig. 1. An unusual diversity of miR-845b superfamily.

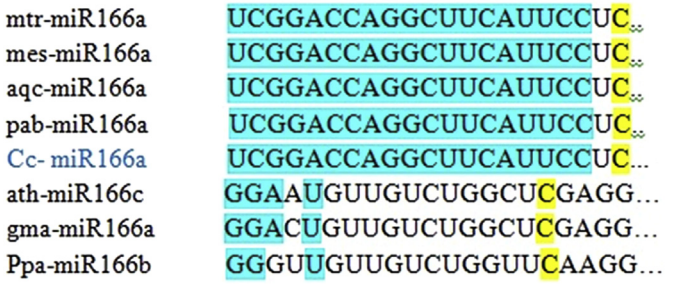

Fig. 2. miR-166 family diversity.

Fig. 3. Expression of Plant U box, CULLIN and F box genes regulated by candidate_81 and candidate_9- candidate_17 respectively in jute RIL75 cells. Immuno Blotting assay for total Plant U box, CULLIN and F box genes expressing above said microRNAs. β-actin was a loading control.

4.1. Role of NBS-LRR proteins in plant defence

The NBS-LRR proteins are normally associated with effect or triggered immunity in which there is a gene-for-gene relationship between the host and the pathogen [27]. The host gene encodes an NBS-LRR protein that mediates recognition, either direct or indirect, of a pathogenesis effector that is encoded by the pathogen. This effector-triggered immunity is normally specific for some but not all races or strains of a pathogen. However, if the NBS-LRR proteins are overexpressed [28], defence can also be induced independently of protein-based recognition mechanisms. An additional potential layer of regulation in this system could involve numerous genes for NBS-LRR proteins in plant genomes [29]. We envision that plants would benefit from low levels of NBS-LRR proteins due to miR-845b in case of low infection pressure or presence of other layers of defence against the pathogen. However, for plants under high infection pressure or without alternatives to the NBS-LRR defence system, the benefit of low expression of NBS-LRR proteins could be effective mechanism of host defence.

4.2. miR-166 superfamily mediated ROS defence

It is well known that ROS such as H_2O_2 play important roles in controlling cellular functions viz., cell differentiation, proliferation, migration, apoptosis and cell death [30]. These cell functional controls are achieved via ROS mediated gene expression regulation [31,32].

In plant system, micro RNAs play a pivotal role against pathogens and accelerate the host defence mechanism. In the present study, we envisioned when *C. capsularis* is infected by *M. phaseolina*, a series of different micro RNAs are activated to target the concerned genes which provide defence against the pathogen. A multi-layered defence was initiated by microRNA to put strong barrier against pathogen.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2016.04.005.

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