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Can-miRn37a mediated suppression of ethylene response factors enhances the resistance of chilli against anthracnose pathogen *Colletotrichum truncatum* L

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ABSTRACT

Pepper anthracnose, caused by *Colletotrichum* species complex is the most destructive disease of chilli (*Capsicum annuum* L.). miRNAs are key modulators of transcriptional and post- transcriptional expression of genes during defense responses. In the present study, we performed a comparative miRNA profiling of susceptible (Arka Lohit-AL) and resistant (Punjab Lal-PL) chilli cultivars to identify 35 differentially expressed miRNAs that could be classified as positive, negative or basal regulators of defense against *C. truncatum,* the most potent anthracnose pathogen. Interestingly, a novel microRNA can-miRn37a was significantly induced in PL but largely repressed in AL genotype post pathogen attack. Subsequent over-expression of can-miRn37a in AL showed enhanced resistance to anthracnose, as evidenced by decreased fungal growth and induced expression of defense-related genes. Consequently, the expression of its three target genes encoding the ethylene response factors (ERFs) was down-regulated in PL as well as in the over-expression lines of AL genotypes. The ability of these targets to be regulated by can-miRn37a was further confirmed by transient co-expression in *Nicotiana benthamiana.* Additionally, the virus-induced silencing of the three targets in the susceptible AL cultivar revealed their role in fungal colonization and induction of *C. truncatum* pathogenicity in chilli. Taken together, our study suggests that can-miRn37a provides a potential miRNA mediated approach of engineering anthracnose resistance in chilli by repressing ERFs and preventing fungal colonization.

1. Introduction

Pepper anthracnose caused by *Colletotrichum* species complex is the most devastating disease of chilli (*Capsicum annuum* L.) in the tropical and sub-tropical regions of the world including India [1]. *Colletotrichum* are ascomycetes fungus that infects the plant through the germination and production of adhesive appresoria followed by penetration into the host epidermis. Under favourable condition, the pathogen colonizes the host tissue by forming dark, sunken necrotic lesions with concentric rings of acervuli leading to pre and post-harvest fruit rot and spots and blights of aerial plant parts [1,2]. Although a number of *Colletotrichum* species are linked to chili anthracnose, *C. truncatum* (formerly referred as *C. capsici*) is ubiquitous and the most destructive species in Indian subcontinent [3]. An estimated yield loss of 29.5%, amounting to US$ 491.67 million has been reported due to chili anthracnose in India [2]. Attempted management of chilli anthracnose through the application of fungicides, biological control agents and host resistance breeding haven't met with tangible success due to environmental toxicity, variation in resistance response, variability in the antagonistic effect of different *Colletotrichum* species and discrepancies in the assessment of disease reaction [1]. In other words, there is no ideal approach for control or alleviation of this disease because the molecular mechanism guiding the incidence of chilli anthracnose is not yet clear.

Plants counter acts against biotic and abiotic factors through a complex network of molecular, cellular and physiological processes [4]. The investigation into the molecular and biochemical mechanism in the interaction between *C. annuum* and anthracnose pathogen have been initiated only recently. Over expression of a defensin gene, *J1-1* provided strong resistance to anthracnose fungus and co-related with up regulated expression of jasmonic acid (JA)-biosynthetic and pathogenesis-related (PR) protein genes [5]. Chilli transgenic lines over expressing a pepper carboxylesterase gene (*PepEST*) exhibited significant resistance against multiple *Colletotrichum* species together with high accumulation of PR-proteins [6]. Besides, the transcript profiling of chilli under compatible and incompatible interaction have identified a number of differentially expressed genes related to JA, ET and SA signalling, *R* and *PR* proteins and defense responsive transcription factors [7]. Alternatively, a *Colletotrichum truncatum cutinase 1 (Ctcut1)* has

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been recognized as a major pathogenicity factor of chilli anthracnose [8]. Yet, the functional role of these genes in the genetic interaction between *C. annuum* and *C. truncatum* is highly ambiguous. Next generation sequencing has emerged as a steadfast and efficient instrument for functional genomics studies and molecular interaction between host and the pathogen [9]. However, a complete delineation of the regulatory network governing the host pathogen interaction depends upon collective contribution of genetic expression at both transcriptional and post-transcriptional levels.

microRNAs are represented by 21–24 nucleotide (nt) small regulatory RNAs transcribed from endogenous miRNA genes in form of a partially double stranded stem-loop primary structure [10]. Mature miRNAs integrate with a functional endonuclease- Argonaute (AGO) forming the RNA induced silencing complex (RISC) and execute the regulation of gene expression by either mRNA cleavage or translational inhibition [11]. Since the discovery of first plant specific miRNAs in 2002, they have been shown to play fundamental roles in numerous biological processes including plant growth, organogenesis, floral differentiation, hormone signalling and stress responses [12]. Recent evidences indicate that miRNAs and the associated RNA interference pathway components are critical elements in the regulation of plant immunity against diverse phytopathogens including bacteria [13,14], viruses [15], fungi [16–18] and oomycetes [19]. miR393 have been identified as the first resistance related miRNA that was induced in response to PAMP peptide *flg22* in *Arabidopsis thaliana* and positively regulate the host immunity against *Pseudomonas syringae* DC3000 by suppressing auxin signalling [13]. miR393 together with miR160 and miR167 creates a negative feedback regulation of the transport inhibitor response 1 (TIR1) gene which is critical to plant innate immunity against fungal phytopathogens [18]. Transgenic rice plants over expressing miR160a and miR398b have reported superior resistance to blast pathogen, *Magnaporthe oryzae* as confirmed by reduced fungal growth, high accumulation of hydrogen peroxide and induced expression of defense responsive genes [17]. Similarly, Osa-miR7695 reported positive regulation of defense against *M. oryzae* by down regulating the expression of elicitor responsive *OsNramp6* (*Natural resistance associated macrophage protein 6*) gene [16]. On the other hand, ata-miR398 and ata-miR733 were described as negative regulators of the PAMP response by preventing callose deposition and cleaving the Cu/Zn superoxide dismutase genes leading to elevated reactive oxygen species (ROS) [14,20]. Additionally, miRNA families such as nta-miR6019 and nta-miR6020 from tobacco, stu-miR1507, stu-miR2109 and stu-miR2118 from potato as well as tomato specific slymiR482f and sly5300 have been identified as resistance regulators by directing the cleavage of nucleotide-binding site leucine rich repeat (NBS-LRR) class resistance (*R*) genes [21–23]. More recently, miR400 targeting pentatricopeptide repeat proteins, miR844 targeting cytidinephosphate diacylglycerol synthase 3 (CDS3) and miR1918 targeting the RING finger proteins have been designated as negative regulator of defense by enhancing the host sensitivity to pathogen attack [24–26]. On the pathogen perspectives, evidences also suggest that translocation of immune regulated miRNAs in interactive organisms may lead to cross-kingdom RNA interference [27]. All these reports clearly suggest a diversified role of miRNAs in the interaction between host and the pathogen towards modulation of immune responses.

Whereas, the regulatory roles of these miRNAs are still poorly understood in many plant species, there is no practical information available about their involvement in *C. annuum-C. truncatum* interaction. A recent study in our lab relating to transcriptome profiling of *C. annuum* post infection with *C. truncatum* revealed 293 expressive miRNA targets (unpublished) suggesting that miRNA mediated post-transcriptional gene silencing machinery could be a prominent determinant in the innate immunity of chilli against *C. truncatum*. Therefore, we performed a systemic sRNA-Seq analysis to determine the relative abundance and differential expression of miRNAs in the susceptible cultivar Arka Lohit and the resistant cultivar Punjab Lal upon *C. truncatum* infection. Further, we also developed transgenic chilli plants over expressing a novel can-miRn37a to evaluate its integrative role in chilli immunity against anthracnose fungus. By examining the virus induced gene silencing (VIGS) lines of the target genes and co-expression of can-miRn37a and corresponding targets in *Nicotiana benthamiana,* we identified three candidate ethylene response factor (ERF) genes that might act as negative regulators for chilli immunity.

2. Materials and methods

2.1. Plant materials, pathogenic infection and deep sequencing

Two *C. annuum* cultivars (cv.) Punjab Lal (PL) and Arka Lohit (AL) with variable sensitivity to the anthracnose pathogen *C. truncatum* (Cot) were used for plant infection. The inoculation was performed in a temperature-controlled growth chamber using a wild type super virulent *C. truncatum* strain MTCC-3414 (collected from Microbial type Culture Collection, Institute of Microbial Technology, Chandigarh, India). Fruits of mature red ripened stage free from micro-organisms were used for Cot inoculation as described previously [7]. The disease severity was determined based on mean percentage lesion size of the fruits. The lesion diameter and fruit size were measured with vernier callipers. Percentage disease index (PDI) value was calculated by dividing the lesion size relative to the overall size of the fruit and the PDI values were transformed to determine the level of aggressiveness with six severity scores on a 0–9 scale. Whole fruit of the control and treated samples were harvested at 0, 3, 5, 7 and 9 days after inoculation (DAI). A set of five fruits were collected for each time point and pooled together for RNA extraction. Total RNA was isolated from the control and treated samples using the Trizol reagent (Invitrogen, Darmsradt, Germany) according to the manufacturer's recommendations. RNA samples with 260/280 nm ratio between 2.0 and 2.1 and RNA integrity number (RIN) $\geq$ 8.0 were used for constructing four small RNA libraries from pooled samples of mock inoculated (AL-Water and PL-Water) and pathogen inoculated (AL-Cot and PL-Cot) fruits using TruSeq Small RNA sample preparation kit (Illumina) following manufacturer's instructions. The tagged libraries were subsequently sequenced using 1 $\times$ 50 bp SE chemistry on an Illumina NextSeq 500 platform at the Xcelris Genomics Pvt. Limited, Ahmadabad, India.

2.2. sRNA-Seq analysis and identification of differentially expressed miRNAs

Illumina sequence reads were filtered for high quality sequences using the FastX tool kit (http://hannonlab.cshl.edu/fastx_toolkit) and adapters were removed using CutAdapt tool. High quality reads were searched with BLAST against the GenBank (http://www.ncbi.nih.gov/GenBank) and Rfam (http://rfam.sanger.ac.uk/) databases to indentify and remove other non-coding RNAs including rRNAs, tRNAs, snRNAs, snoRNAs etc. Unmapped 18–26 nt clean sRNA reads were subjected to BLASTn search against the mature sequences in the miRBase (http://www.mirbase.org) to identify known miRNAs. The identified miRNA sequences were mapped to *Capsicum annuum* L. reference genome (http://peppersequence.genomics.cn) and their expression and distribution were analyzed using SOAP software. The unannotated tags that did not match any sequence from miRbase were subjected to novel miRNA prediction using miRDeep-P [28] and miRCat (http://srna-workbench.cmp.eua.ac.uk/tool) sRNA workbenches. To identify differentially expressed miRNAs across *C. truncatum* infected chilli fruits, we first normalized the frequency of miRNAs in the four libraries as transcript per million (TPM) i.e. actual miRNA counts/total count of clean reads $\times$ 1,000.000. The log2 fold change and *P* values were calculated from the normalized expression values as described previously [29]. A *P* value $\leq$ 0.01 and log2 fold change $\geq$ 2.0 was taken as cut off to determine the significance of difference in miRNA expression.