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Uncovering roles of microRNAs in regulation of curcumin biosynthesis in turmeric (*Curcuma longa* L.)

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ABSTRACT

Curcuma longa L. is an important spice with various therapeutically active secondary metabolites in its edible rhizomes and curcumin is the most important among them. Secondary metabolite synthesis in plants is reported to be a highly regulated and *microRNAs* are a class of small endogenous non-coding RNAs (sRNAs) that could play a major role in gene regulation. *miRNA* mediated regulation of biosynthesis of curcumin is an unexplored area. We have identified, through transcriptome analysis, about 29 *miRNAs* that showed differential expression with respect to curcumin in turmeric accessions with contrasting curcumin content. Expression of one of the conserved *miRNAs viz.*, miR319 showed a negative correlation to curcumin when plants were grown under different light regimes favouring differential curcumin accumulation in rhizomes. This *miRNA* is a potential candidate for further studies on regulation of biosynthesis of curcumin.

Keywords: *miRNA*, RNA-seq, curcuminoids, biosynthetic pathways

INTRODUCTION

Turmeric (*Curcuma longa* L.) is an economically important medicinal plant belonging to the family Zingiberaceae. Curcuminoids, the biologically active phenylpropanoid derived from turmeric, is the focus of attention now a days due to its immense therapeutical benefits. The curcumin content in plants is subject to environmental variations and growth conditions and is the major challenge faced in its industrial exploitation. Understanding the regulation of its biosynthesis is thus essential in order to achieve stable and higher levels of curcumin irrespective of external conditions. The highly regulated nature of curcumin biosynthesis was evident from our transcriptome studies in turmeric (Sheeja *et al*., 2015; Deepa *et al*., 2017). We could identify several genes and transcription factors (TFs) that showed differential expression with respect to curcumin and we are currently working on cloning and sequencing of *bonafide* genes and transcription factors of the pathway. The upstream sequences of some of those genes revealed several MYB TF binding domains (Deepa *et al*., 2017). These domains are expected to be the regulatory sites for small RNAs like miRNAs. In many of the studies on biosynthetic pathways, *miRNAs* were identified to regulate gene expression (Ong and Wickneswari, 2011). We had identified conserved, non conserved and novel miRNAs for the first time in turmeric through *in silico* prediction and Illumina deep sequencing (Santhi and Sheeja, 2015; Santhi *et al*., 2016) and in the present study, we have made an attempt to verify the role of *miRNAs* in regulation of curcumin biosynthetic pathway through an Illumina based deep sequencing of two accessions contrasting in curcumin content. We also studied gene expression of a specific *miRNA* reported to regulate biosynthesis of secondary metabolites in plants (*miR319*), under different light regimes favoring differential accumulation of curcumin (Deepa *et al*., 2017).

MATERIALS AND METHODS

Plant material

Rhizomes of a high curcumin variety (IISR Prathibha) and a low curcumin accession (Accession 449) were used in the study. For Illumina deep sequencing, total RNA from rhizomes of IISR Prathibha and accession 449 were isolated according to the method described by Deepa *et al*. (2014). The purity of total RNA was analyzed with Agilent 2100 Bioanalyzer. Small RNA cDNA library was constructed using Illumina small RNA sample preparation kit according to manufacturer's protocol and subjected to Illumina sequencing. Total RNA isolation, small RNA cDNA preparation and sequencing were outsourced. For evaluating

miRNAs (*miR319*, *miR828* and *miR858*), IISR Prathibha grown under different shade nets (red, green and white) and open condition, were collected from the field of ICAR-Indian Institute of Spices Research (11.2994◦N, 75.8407◦N), Kozhikode, Kerala.

Identification and expression profiling of miRNAs

Small RNA trimming, pre-processing and adaptor removal were performed with CLC genomic workbench. High quality reads were further analyzed to extract small RNAs, by annotated against miRBase to identify conserved miRNAs. Clean high-quality reads were mapped to curated plant miRNA database to identify differentially expressed miRNAs using small RNA seq and RNA seq tools in CGWB. The number of reads mapped to each miRNA species was considered as a measure of expression. Count expression values were normalized using quantile normalization and the normalized expression values were subjected to Kal's Z test on proportions to get significant fold change values at $p<0.05$.

miRNA cDNA synthesis and validation by stem loop RT-PCR

Total RNA was extracted from rhizome of IISR Prathibha grown under different shade net conditions (Deepa *et al*., 2014) followed by removal of DNA using *DNase* I (Thermo Scientific) at 37ºC for 30 min and inactivated by adding 1 µL 50 mM EDTA (Himedia) at 65°C for 10 min. Primer design, cDNA preparation and end-point PCR were performed as previously reported (Varkonyi-Gasic *et al*., 2007) and resolved in 4% agarose (Sigma) gel under constant current of 90V till the bromophenol blue dye (Himedia) front migrated to the bottom of the gel. The gel was visualized under UV using gel documentation system (Syngene).

Real time PCR analysis of *miRNAs*

Total RNA isolation, *DNase* treatment and cDNA synthesis were performed as described previously. To check the expression fold change in *miRNAs*, real-time PCR was employed using QuantiFast SYBR Green PCR Kit (Qiagen) on Rotor Gene-Q real-time PCR (Qiagen). All reactions were performed three times. qPCR parameters were 95° C for 5 min, 40 cycles of 95° C for 10 s, 60° C for 30 s. Melt curve analysis was performed at 70 °C-95°C to check the specificity of PCR products. Relative gene expression was calculated using 2^DDCt method. *18S rRNA* was used as the endogenous control.

Estimation of curcumin content

The rhizomes were dried and ground to a fine powder and curcumin content was analysed by the procedure as per American Spice Trade Association (ASTA) (1968).

RESULTS AND DISCUSSION

Identification of conserved miRNAs

Twenty nine conserved *miRNAs* could be identified from small RNA libraries of a low curcumin (2.11%) Accession 449 and a high curcumin (6.52%) variety IISR Prathibha and the relative gene expression was correlated to curcumin content. On the basis of deep sequencing data, miRNAs with fold expression greater than 2.0 were chosen that included 14 miRNAs that were down-regulated and 15 *miRNAs* that were up-regulated. The expression levels of *miR168b*, *miR167a*, *miR156q*, *miR156e*, *miR164d*, *miR396e* and *miR396h* were significant. These *miRNAs* could probably be involved in regulation of biosynthesis and need to be confirmed through expression studies.

Expression profiling of *miRNAs* under different light regimes

Phenylpropanoid biosynthetic pathway is highly regulated by spatial and temporal regulation of specific genes, and also in response to both endogenous (metabolic and hormonal) and environmental (light, pathogen attack, cold, etc.) stimuli (Weisshaar and Jenkins, 1998). MYB transcription factors are reported to play an important role in regulation of phenylpropanoid biosynthesis in plants (Dubos *et al*., 2010). Curcumin is a phenylpropanoid derivative so it is envisaged that MYB transcription factors may have a role in the regulation of curcumin biosynthesis too. We have already identified and shortlisted about 20 MYBs from transcriptome studies conducted involving samples with contrasting curcumin contents (unpublished data). MYBs are in turn down regulated by small RNA molecules like miRNAs. These results encouraged us to check the *miRNA* mediated regulation of MYB TFs *vis a vis* curcumin content. IISR Prathibha grown under shade nets showed a differential accumulation of curcumin based on the shade net colour (Deepa *et al*., 2017). Curcumin contents under green (4.93%) white (4.84%) red (4.45%) and open condition (5.22%) were analysed and correlated to expression of *miRNAs*. Three conserved miRNAs *viz., miR319* (Santhi *et al*., 2016; Santhi, 2018), *miR828* (Deng *et al*., 2017) and *miR858* (Sharma *et al*., 2016), already reported to play big roles in phenylpropanoid metabolism and regulate MYB transcription factors were amplified under the above experimental conditions. All three *miRNAs* could be amplified via end point PCR (Fig. 2, 4, and 6) as well as real time PCR (Fig. 1, 3, and 5). However, only *miR319* showed a negative correlation under all three conditions with maximum expression under red shade net and lowest

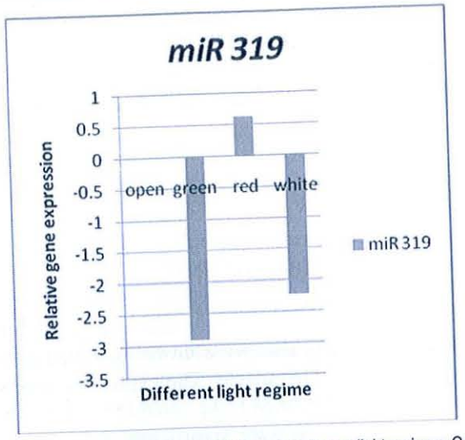

Fig. 1: Real time PCR analysis of miR319 under different light regimes, Control: IISR Prathibha grown in open condition

Fig. 2: Amplification of *miR319* in rhizomes under different light regimes: Lane 1-10bp ladder, Lane 2-Control (Open), Lane 3-Red shade net, Lane 4-Green shade net, Lane 5-White shade net

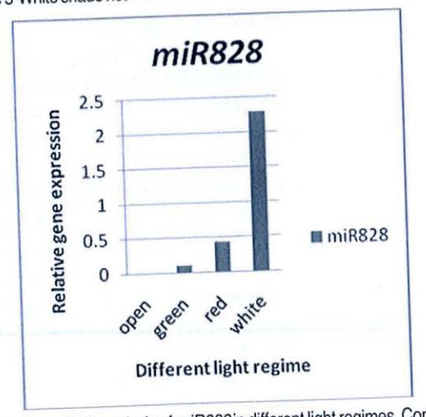

Fig. 3: Real time PCR analysis of *miR828* in different light regimes, Control: IISR Prathibha grown in open condition

Fig. 4: Amplification of miR828 under different light regimees regimes Lane 1-10bp ladder, Lane 2-Control (Open), Lane 3-Red shade net, Lane 4-Green shade net, Lane 5-White shade net

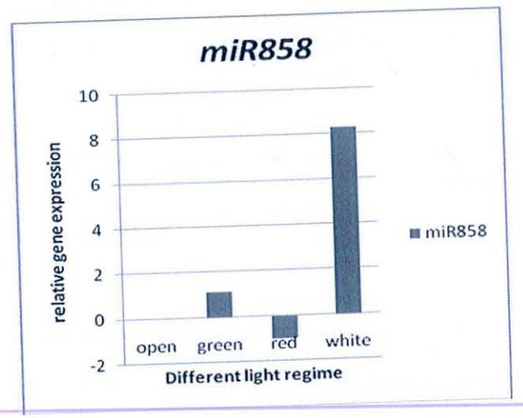

Fig. 5: Real time PCR analysis of *miR858* under different light regimes, Control: IISR Prathibha grown in open condition

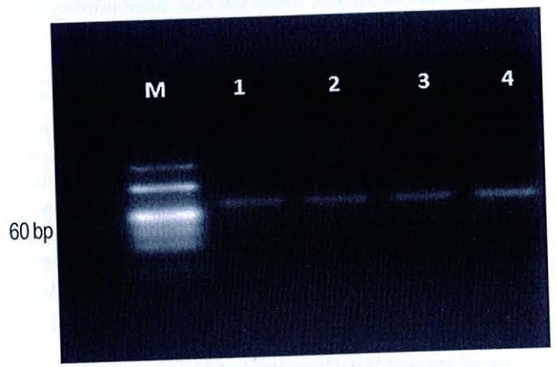

Fig. 6: Amplification of miR858 in different light regime
Lane 1-10bp ladder
Lane 2-Control (Open)
Lane 3-Red shade net
Lane 4-Green shade net
Lane 5-White shade net

under green shade net conditions. It could be presumed that miR319 could probably be an inhibitor of MYBs that are activators of curcumin biosynthesis. A similar work conducted by Thiebaut *et al*. (2012), on *miR319* also reports the targeted cleavage of the gene *GAMyb* during cold stress in sugarcane. However, more studies are required to confirm the results of our study.

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