Research Article

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Molecular cloning and characterisation of a novel putative MYB-related transcription factor (*ClMYB1R1*) from *Curcuma longa* L.

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Abstract

Turmeric (*Curcuma longa* L.) is gaining immense global importance due to the presence of curcumin that contributes to its immunomodulatory properties. Curcumin, a phenylpropanoid derivative, is the most important secondary metabolite present in turmeric rhizomes and understanding the regulatory mechanism of curcumin biosynthesis is hence important. In plants, activator and repressor type transcription factors (TFs) of the myeloblastosis (MYB) family have been found to regulate the biosynthesis of secondary metabolites. MYB TF genes of the R2R3 class were identified by RNA-Seq based approach from turmeric. The present study involves RNA seq based identification, cloning and characterisation of a novel MYB TF of the 1R class. Gene-specific primers were used to amplify 1R MYB from the rhizome, and it was sequenced to give a full-length gene of 1056 bp. The identified MYB gene was designated as *ClMYB1R1* and consisted of a 693 bp open reading frame (ORF) which encodes a 230 amino acid length protein with a molecular mass of 25 kDa and pI 9.51. The protein was predicted to consist of a single helix-turn-helix MYB-like motif and other highly conserved residues. *ClMYB1R1* expression varied in different genotypes and tissues. UV-C light was found to upregulate, while NaCl and nutrient stresses down-regulated the expression. The expression showed a positive correlation with a candidate pathway gene *ClPKS11* under all of the above experimental conditions, indicating the putative role of *ClMYB1R1* in regulating curcumin biosynthesis.

Keywords: Curcumin, MYB, phenylpropanoid pathway, transcription factor, turmeric

Introduction

MYB proteins are a family of transcription factors (TFs) abundant in plants (Dubos *et al.*, 2010). They are involved in various biological processes, such as circadian rhythm, defence and stress responses, cell fate and identity, seed and floral development, and regulation of primary and secondary metabolism in plants (Dubos *et al*., 2010; Jin *et al*., 1999; Liu *et al*., 2015; Xu *et al.,* 2015; Ramya *et al*., 2017). MYB TFs also play an important role in regulating biosynthetic pathways of secondary metabolites (Huang *et al.,* 2016; Deng *et al*., 2020; Cao *et al*., 2020). They are categorised into different classes based on the number of repeats on their DNA binding domain, which include 1R (MYB-related), R2R3, 3R and 4R classes (Stracke *et al*., 2001). Though a large number of studies were carried out on the R2R3 class, studies on 1R MYB-related proteins in plants are very few (Du *et al.*, 2013). Already several R2R3 MYBs were identified from turmeric rhizome transcriptome (Sheeja *et al.,* 2015) and analysed the expression of a few of them. However, no information on the 1R class is available yet in turmeric. 1R MYBs possess a single MYB domain and are predominantly known as transcriptional regulators in plants. This subfamily is further divided into several subgroups, including CCA1-like, I-box-binding-like, TBP-like, TRF-like, CPC-like and other MYB-related proteins (Yanhui *et al*., 2006). They act as circadian oscillators in developmental modulation (Wang and Tobin, 1998; Kuno *et al.*, 2003, Schaffer *et al.*, 1998), regulate

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hormone balance (Klinge *et al.*, 1997) and are involved in stress responses (Cheng *et al.*, 2013). 1R MYB TFs are also reported as potential regulators of phenylpropanoid biosynthesis in many plants (Feller *et al*., 2011; Yi *et al*., 2010; Zhang *et al.*, 2009; Zhu *et al.*, 2009; Nemie-Feyissa *et al.*, 2014; Dubos *et al*., 2008; Matsui *et al*., 2008). However, reports on 1R MYB TFs regulating the phenylpropanoid pathway in turmeric is not yet available.

The current study reports the isolation and cloning of a novel MYB-related TF, *ClMYB1R1* of the 1R class from turmeric. The putative role of this gene in curcumin biosynthesis is evaluated through co-expression analysis under different experimental conditions *vis-a-vis* a putative candidate gene of the pathway. We have also characterised the *ClMYB1R1* using *in silico* approaches.

Materials and methods

Plant materials, cDNA library construction, Illumina sequencing and MYB transcript annotation

Rhizomes of four-month-old IISR Prathibha (curcumin content- 6.52%) grown in the field under normal management conditions (TC) (Srinivasan *et al*., 2016) and the same variety grown in a pot in the greenhouse under nutrient stress (curcumin content-1.54 %), depriving all inputs (TS) were used for isolation of total RNA. Rhizomes were harvested and immediately stored in 'RNAlater' (Sigma) at -20^{0}C until RNA isolation. For Illumina sequencing, RNA was isolated as per the method described by Deepa *et al*. (2014), and the purity was analyzed with Agilent 2100 Bioanalyzer and samples with RIN >7.5 were used for RNA-Seq analysis. cDNA library construction and sequencing were carried out at SciGenome Labs, Kochi. Transcriptome library was constructed using Illumina's TrueSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) following manufacturer's instructions and was sequenced on Illumina HiSeq 2500 platform by paired-end sequencing. The bioinformatic analysis was carried out at SciGenom Labs Pvt. Ltd., Cochin, Kerala.

The assembled transcripts were annotated using in-house pipeline programs for *de novo* transcriptome assembly and compared with NCBI non-redundant protein database using BLASTX program to identify differentially expressed MYB related TFs (Bio Project ID-PRJNA698442). Among the upregulated MYB related transcripts in TC, one belonging to class 1R with maximum fold change and a complete ORF was chosen for the study.

Comparative analysis of gene expression of *ClMYB1R1*

a. Different accessions and tissues (Field)

Four-month-old rhizomes from IISR Prathibha (curcumin content: 5.4%) and Accession No. 200 (curcumin content: 3.4%) grown under normal conditions (Srinivasan *et al*., 2016) were used for comparing the expression pattern of *ClMYB1R1*. Four distinct tissues (rhizome, leaf, pseudostem and root) from four-month-old IISR Prathibha were taken for analysing the tissue-specific expression of *ClMYB1R1*.

b. UV-C and NaCl stress (*in vitro*)

Rhizomes of turmeric variety IISR Prathibha were cultured *in vitro* (Babu *et al*., 2016). Two-month-old aseptically grown *in vitro* plantlets with 3-4 leaves about 10 cm in length and attached roots were used for the study. For NaCl stress, plantlets were grown in 200 mM NaCl for one week (PS). UV-C stress was imposed by exposing plantlets to UV-C rays for 15 minutes (PU). Gene expression was compared with control under normal conditions (PC1 & PC2, respectively).

c. Nutrient stress (Greenhouse)

Four-month-old rhizomes from IISR Prathibha were grown in pots in a greenhouse and subjected to nutrient stress by withholding nutrients (PN; curcumin 2.2%) was compared with control maintained under normal management conditions (PC3; curcumin 5.0%) (Srinivasan *et al*., 2016).

Cloning of 1R MYB related TF

ClMYB1R1 was amplified from both genomic DNA and cDNA from rhizomes of four-month-old plants of IISR Prathibha grown under normal conditions in the field. The primers used for the amplification were designed based on the transcripts retrieved from transcriptome data. PCR was performed using single-stranded cDNAs (500 ng μL^{-1}) as the template for 30 cycles under the following

conditions: 95ºC 30s, 58ºC 30s followed by a final step @72ºC 60s. The amplified product was purified (Qiagen, USA) and cloned into the PMD 20T vector (Promega, USA), transformed into *E. coli* strain DH5α and sequenced at Agri Genome Pvt. Ltd. The sequences were matched with those retrieved from RNA-Seq data using the basic local alignment search tool (BLAST).

Analysis of physico-chemical properties and phylogenetic analysis

MYB-related gene sequences were decoded to obtain the information on the corresponding protein. The primary structural parameters, molecular weight, theoretical pI and amino acid composition of the deduced ClMYB1R1 were determined using ExPASy ProtParam. PROSITE analysis was performed to check for the MYB domain and other additional domains in the deduced protein. Alignment of ClMYBIR1 protein with other structurally related MYB-related proteins was performed using the ClustalW program, and a phylogenetic tree was constructed using MEGA 7.0 (www.megasoftware.net) with default parameters.

Secondary structure prediction

The properties like α-helix, β-sheet, and turn of amino acid sequences of ClMYB1R1 protein was predicted using secondary structure prediction tool PSIPRED (McGuffin *et al*., 2000) and self-optimized prediction method with the alignment of SOPMA analysis (Geourjon *et al*., 1995).

Three-dimensional structure prediction

The three-dimensional structure of ClMYB1R1 was predicted by SWISS Modelling. Different models were generated using protein database (PDB) homologs as templates, and one model with a maximum per cent similarity was selected. Ramachandran plot was constructed to evaluate the stereochemistry of the optimized model.

Expression Analysis of *ClMYB1R1*

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using cDNA from all treatments and controls (PS, PU, PN and PC1-PC3) for analysing the expression of *ClMYB1R1*. Experiments were performed by pooling three biological replicates, and three technical replicates were taken per sample. Quantitative PCR amplification was performed in 20 µL reaction containing 1 µL single-stranded cDNA (250 ng), 0.5 µL forward and reverse gene primers (10 µm) and 10 µL of Quantifast SYBR Green 2X master mix. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Actin and ubiquitin were used as the endogenous control (Deepa, 2018; Santhi, 2018). A novel putative gene *ClPKS11* involved in curcumin biosynthesis (Deepa *et al.,* 2017) was used to compare expression profiles in all the experiments.

Estimation of curcumin content

The curcumin content of all samples was analyzed as per American Spice Trade Association (ASTA) procedure. One gram of powdered dried rhizomes was added to 75 mL of acetone and reflexed for 1 hr. After cooling, it was filtered and made up to 200 mL with acetone. The extract (1 mL) was made up to 100 mL in a volumetric flask, and absorbance was checked at 425 nm with acetone as blank.

Results and discussion

Transcriptome sequencing and annotation of MYB TFs

De novo assembly of rhizome-specific transcriptomes of TC and TS produced a total of 138,015 contigs with the longest transcript length of 37,043 (bp) and mean GC per cent of 46. Transcripts were blasted against Plant Transcription Factor Database (Plant TFDB) with an e-value cut off 10^{-10} Among these, about 86 contigs were found to belong to MYB related TF class. A candidate MYB *viz., ClMYB1R1* with 2.14-fold up-regulation in TC with a full-length ORF was chosen for study.

Expression profiling of 1R MYB TF

The phenylpropanoid biosynthetic pathway is controlled by several genes and TFs (Cavallini *et al.*, 2015) and MYB TFs are the major regulation players (Dubos *et al*., 2010). Since curcumin is a phenylpropanoid derivative, it is assumed that MYB TFs may have a role in regulating biosynthesis. We had already identified R2R3 MYBs by RNA Seq from turmeric (Sheeja *et al*., 2015), and many TFs belonging to the 1R class could be identified in the

present study. Among those, one transcript *viz., ClMYB1R1* showed 2.14 fold differential expression under high curcumin conditions (TC) compared to that under stress TS (lower curcumin) was chosen for study. Full-length ORF of 693 bp could be retrieved from the transcriptome data for this gene.

To verify the role of the gene in the biosynthesis of curcumin, co-expression analysis was conducted under different experimental conditions *vis-a-vis* the putative candidate gene *ClPKS11* (Deepa *et al*., 2017). IISR Prathibha (curcumin 5.4%) showed 2.6 fold higher expression than Accession no. 200, having 3.4 per cent curcumin (Fig. 1). Among the different tissues analysed, maximum expression of *ClMYB1R1* and *ClPKS11* was observed in rhizomes, followed by the pseudostem, root and leaf (Fig. 2). Tissue specificity or dramatically different expression levels of transcription factors in different tissue types allow differential metabolite accumulation. In the case of tomatoes, tissue specificity of transcription factors involved in the fruit ripening process was identified by qRT-PCR

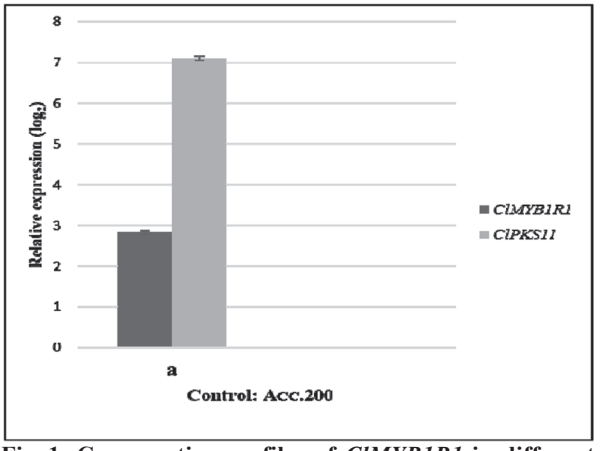

Fig. 1. Comparative profiles of *ClMYB1R1* in different accessions. a) IISR Prathibha (curcumin 5.4%); control: Acc. 200 (curcumin 3.4%). Error bars represent standard errors of the mean from three technical replicates

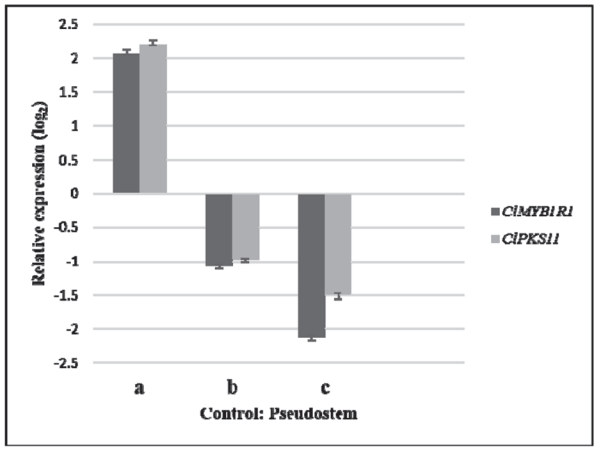

Fig. 2. Tissue-specific expression pattern of *ClMYB1R*. a: rhizome; b: root; c: leaf; control: pseudostem. Error bars represent standard errors of the mean from three technical replicates

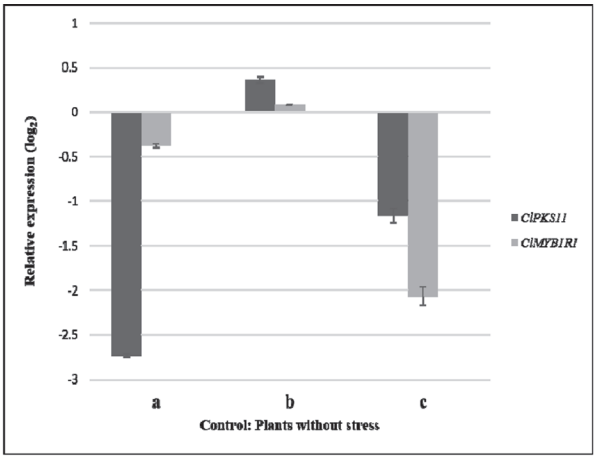

Fig. 3. qRT-PCR analysis of *ClMYB1R1* under different stress conditions. a: NaCl stress (200 mM NaCl for 1 week); b: UV-C (15 minutes); c: Nutrient stress (withholding nutrients). Error bars represent standard errors of the mean from three technical replicates

analysis (Rohrmann *et al*., 2012). The expression profiles of *ClMYB1R1* correlated well with that of the putative marker gene *ClPKS11*, a positive regulator of curcumin biosynthesis in all the above cases (Fig. 1, Fig. 2 and Fig. 3).

Plantlets treated with 200 mM NaCl under *in vitro* conditions (PS) compared to PC1 showed downregulation of *ClMYB1R1*. A MYB related homolog (*LlMYB3*) identified from tiger lily was functionally validated as a positive regulator in plant stress tolerance (Yong *et al.,* 2019). A MYB-related gene, *FvMYB1,* was found to increase remarkably in response to salt treatment and exhibited tolerance to salt stress in transgenic tobacco (Li *et al.*, 2016), and *AmMYB1* from a mangrove tree was also found to confer salt tolerance in transgenic tobacco (Ganesan *et al.*, 2012). Whereas in the case of

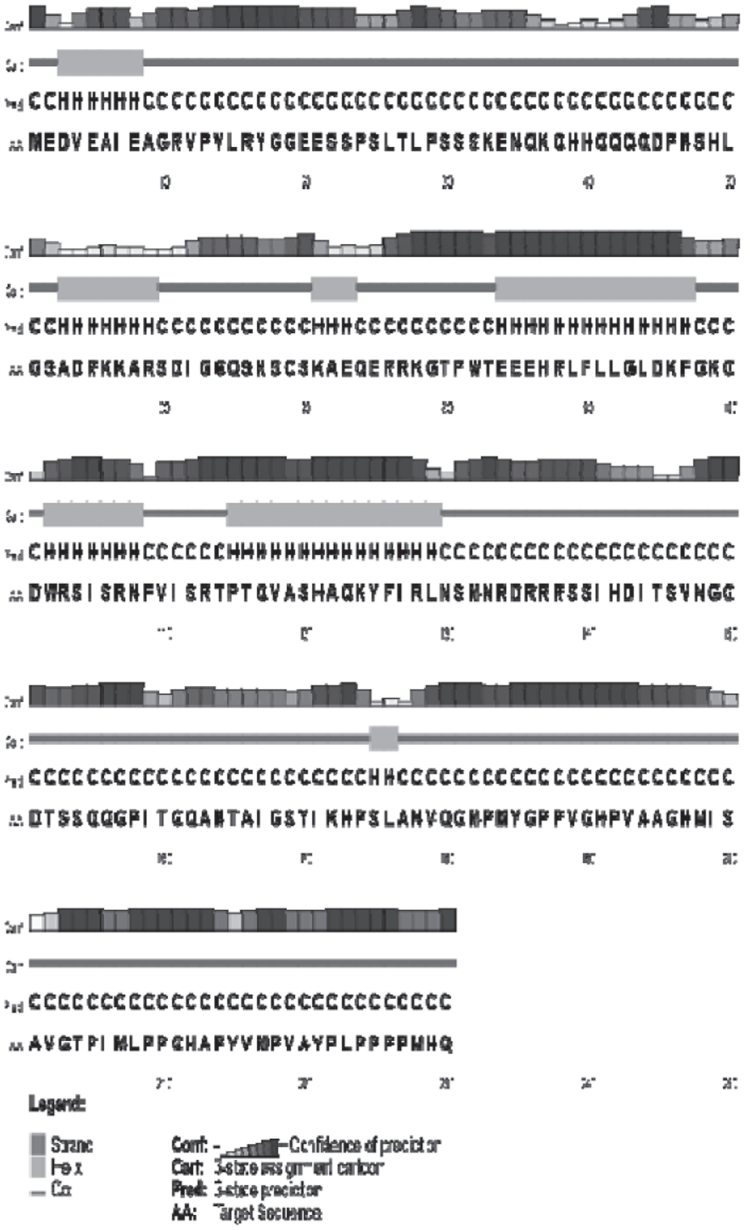

Fig. 4. Prediction of *the* secondary structure of *ClMYB1R1* by PSIPRED analysis

ClMYB1R1, down-regulation of the gene was observed under salt stress.

The *in vitro* plantlets exposed to UV-C light (PU) showed a slight increase in *ClMYB1R1* expression (Fig. 3). The impact of UV-A and UV-B on phenylpropanoid biosynthesis and expression of MYB TFs are well studied in plants (Jin *et al.*, 2000; Czemmel *et al*., 2017). UV-C exposure resulted in a change in gene expression and metabolism and induced biosynthesis of resveratrol and its derivates in grapevine (Petit *et al*., 2009, Chou *et al*., 1978, Wang *et al.*, 2013). Mutants of chalcone synthase gene in *Arabidopsis* were sensitive to high irradiance UV light and resulted in low content or absence of kaempferol forms in leaves (Li *et al*., 1993).

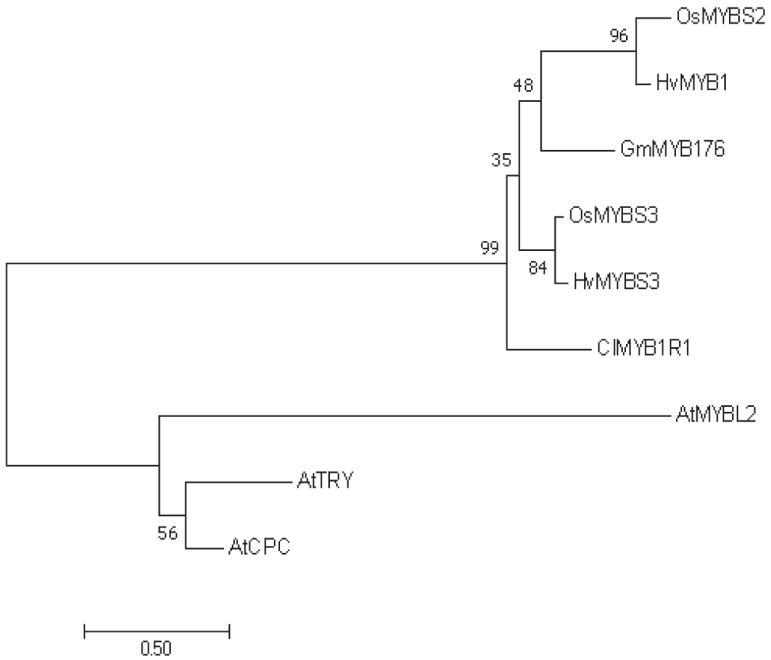

Fig. 5. Phylogenetic tree constructed using amino acid sequences of ClMYB1R1 and other IR class members. Accession numbers of the sequences taken for the comparison are, AtMYBL2 (AEE35154), AtTRY (AED96321), AtCPC (AEC10691), GmMYB176 (ABH02865), OsMYBS2 (AAN63153) and OsMYBS3 (AAN63154)

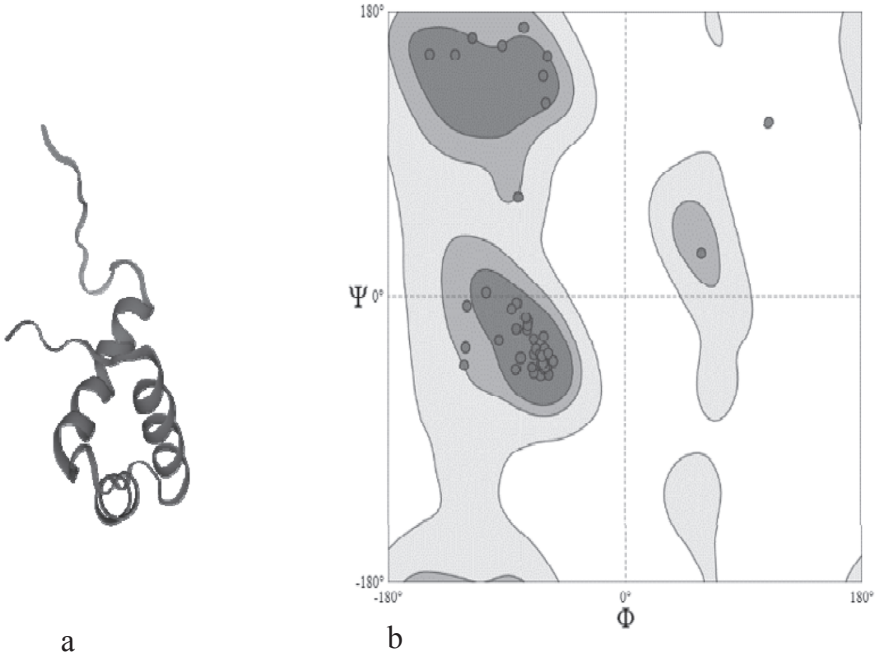

Fig. 6. (a) Predicted 3D structure of *ClMYB1R1* by Swiss modelling with a single MYB domain. (b) The stereochemical validation of the hypothetical model by Ramachandran plot analysis

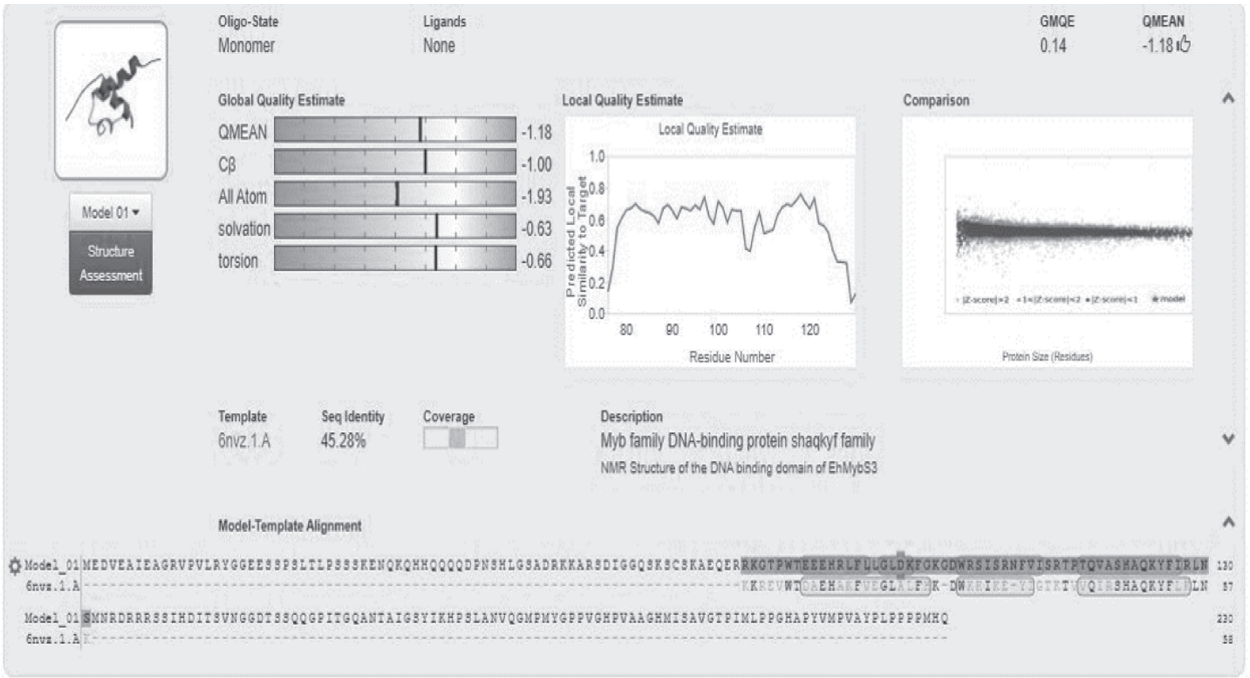

Fig. 7. Quality estimation (GMQE, QMEAN, local quality estimate, and comparison plot) of *ClMYB1R1* based on homology modelling prediction report

The expression of *ClMYB1R1* was found to be down-regulated under nutrient stress. However, in *Arabidopsis thaliana,* a MYB TF encoded by PHR1 that participates in the Pi starvation signalling system was found to be upregulated during phosphate stress (Rubio *et al*., 2001). Also, OsPTF1 was induced during Pi starvation in roots and imparted tolerance to Pi starvation in transgenic rice (Yi *et al*., 2005). It could be thus presumed that *ClMYBR1* showed a genotype-specific, tissue-specific expression pattern and was influenced by UV-C, salt and nutrient stresses. A positive correlation with a candidate gene for curcumin biosynthesis under all the above experimental conditions indicate that it might be involved in the regulation of the pathway too. However, further studies are needed to prove this concept.

Cloning and characterisation of *ClMYB1R1*

ClMYB1R1 was amplified from cDNA as well as genomic DNA, and the full-length gene was cloned. The primers used for amplification are given in Table 1. The 1056 bp full-length cDNA contained a 693 bp ORF with a 171 bp 3' UTR downstream from the stop codon and a 192 bp 5' UTR upstream of the start codon. Gene organisation showed that the *ClMYBIR1* DNA sequence had two exons (260 and 433 base pairs) and one intron (180 base pairs)

Table 1. Primer sequences for full length and qRT-PCR amplification

Sl. No.	Description	Sequence 5‘- 3‘	Tm
	Full length primers		
1.	*ClMYBIR1*FP	AGGGATGGAGGACGTGGAGCCA	60
2.	*ClMYBIR1*RP	ACTATTGGTGCATTGGTGG	58
	qRT PCR Primers		
3.	r*ClMYBIR1FP*	GCACCACCAACAACAACAAG	55
4.	r*ClMYBIR1*RP	GTCCATGGCGTTCCCTTC	55

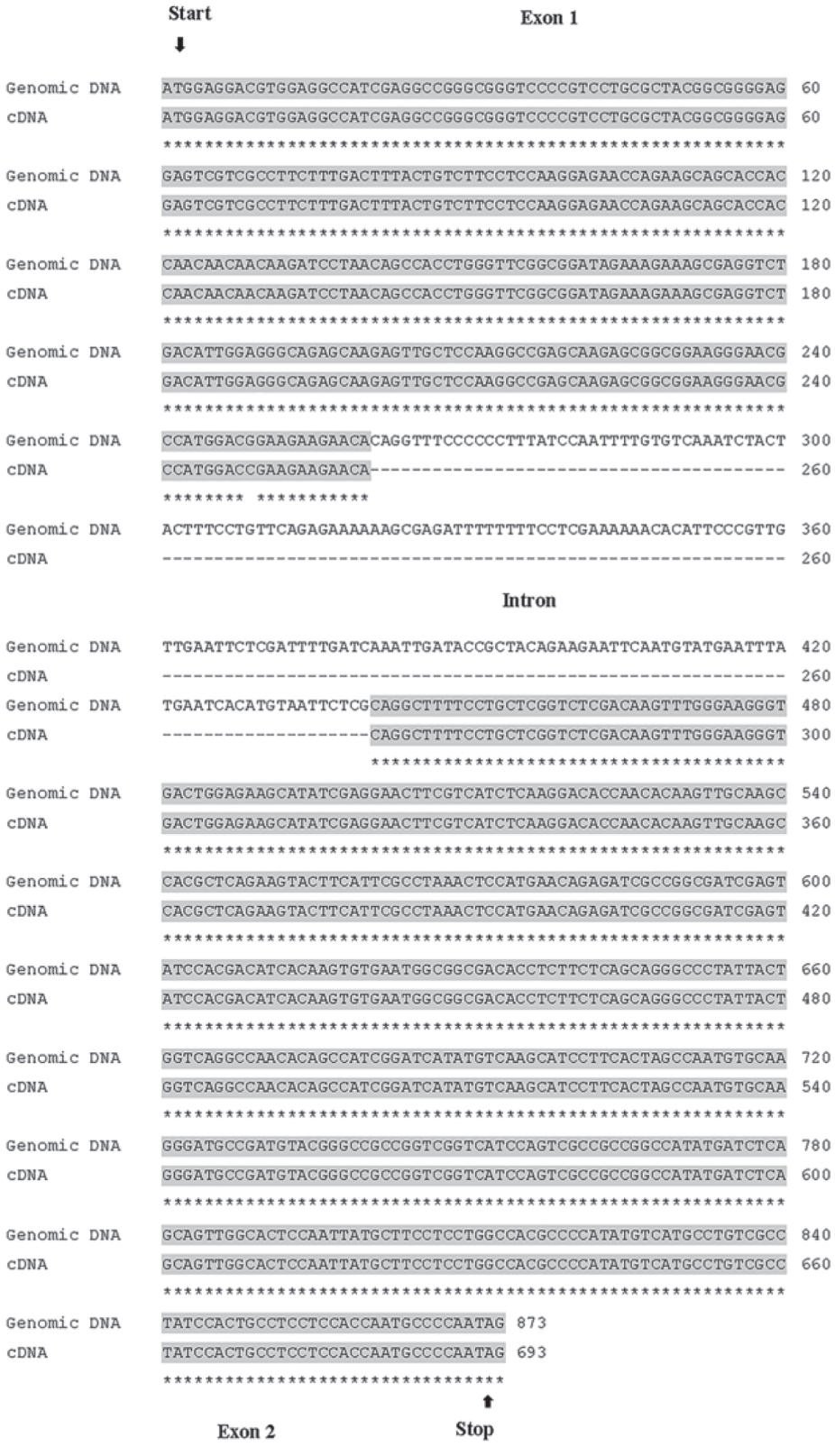

Fig. 8. Exon intron organisation of *ClMYB1R1* generated by comparing genomic DNA and cDNA sequences

(Fig. 8). The protein sequence of putative 1R MYB indicates a molecular weight of 25 kDa with 230 amino acids and a theoretical PI of 9.51.

From the phylogenetic tree, it was evident that the *ClMYB1R1* clustered along with other MYB related TFs with a DNA binding SHAQYF motif (Rubio-Somoza *et al.,* 2006) (Fig. 5). The MYB TFs are divided into five sub-families. The CCA1-like subfamily is the largest of the five subgroups. Almost all members of this subfamily contain the conserved motif SHAQK(Y/F)F within the MYB repeat (Yanhui *et al*., 2006).

The secondary structure of ClMYB1R1 proteins indicated 67 per cent random coiling, followed by 17 per cent extended strand and 10 per cent alpha helices (Fig. 4). The Myb-like domains (amino acids 98-158) of ClMYB1R1 with a more random coil indicates better tolerance to mutations than the helical areas (Chou and Fasman, 1978). The predicted 3D structure showed 45 per cent similarity to MYB family DNA-binding protein SHAQKYF family protein (Fig. 6a), indicating that they belong to the IR class and lie within acceptable predefined limits of similarity (Waterhouse *et al.,* 2018). Ramachandran plot indicated that 98.0 per cent of the residues were in the favoured region and 2 per cent of the residues were in the allowed region, endorsing the predicted model (Fig. 6b) to be acceptable (Ramachandran *et al.*, 1963). In the Swiss modelling analysis QMEAN Z-score of the predicted protein was found to be -1.18, and it indicates the degree of nativeness of the structural features within the model (Fig. 7). QMEAN Z-score value of approximately zero specifies superior quality between the modelled structure and experimental structures, and a score of -4.0 or below indicate poor quality of the proposed model (Benkert *et al*., 2011). In our case, a value of -1.18 was obtained, indicating that the proposed homology model is reliable and acceptable.

Conclusion

Thus, the novel 1R MYB related TF identified in the present study could be a putative positive regulator of curcumin biosynthesis and is the first report. Further functional studies are needed to validate the observation.

Acknowledgement

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