



Novel bHLH and WD40 transcription factors from turmeric (*Curcuma longa* L.) as putative regulators of curcumin biosynthesis

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Abstract

Turmeric, the golden spice belonging to the family Zingiberaceae, is enriched with biologically active curcuminoids composed of curcumin, demethoxycurcumin and bisdemethoxycurcumin. Curcuminoids are phenylpropanoid derivatives, and the biosynthetic pathway is controlled by several transcription factors (TFs). bHLH, WD40 and MYB TFs are the most important TFs regulating phenylpropanoid biosynthesis in plants. Through comparative transcriptome analysis of high and low curcumin germplasm accessions, 20 TFs belonging to the classes bHLH, WD 40, NAC, WRKY and bZIP, which showed differential expression with respect to curcumin, were identified. Among these, two bHLH and one WD40 TFs showed maximum comparative fold change and negative correlation *vis-a-vis* curcumin content in quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The results of comparative transcriptome and qRT-PCR analyses were in congruence, indicating their putative role as negative regulators.

Keywords: Curcumin biosynthetic pathway, qRT-PCR, transcription factors, transcriptome

Introduction

Curcuminoids are biologically active compounds present in rhizomes of turmeric (*Curcuma longa* L.). Curcuminoids are a mixture of curcumin, demethoxycurcumin and bisdemethoxycurcumin. In recent years curcumin has attracted significant attention due to its health-promoting effects, such as protection against cancer, heart diseases (Wongcharoen and Phrommintikul, 2009) and other chronic human disorders (Mantzorou *et al.*, 2018). Moreover, curcumin has also been used as a natural replacement for synthetic food colourants (Ismail and Sakr, 2016) and is an integral part of religious ceremonies (Chattopadhyay *et al.*, 2004).

Curcuminoids are a class of secondary metabolites synthesised through complex biosynthetic pathways that involve structural genes responsible for the formation of final metabolite and regulatory genes that regulate their expression.

Curcuminoid biosynthesis involves several enzyme-coding structural genes such as phenylalanine ammonia lyase (*PAL*), cinnamate 4 hydroxylase (*C4H*), 4-coumaryl CoA ligase (*4CL*), diketide CoA synthase (*DCS*) and curcumin synthases 1, 2, and 3 (*CURS1*, *CURS2*, *CURS3*) (Katsuyama *et al.*, 2009a; 2009b). Earlier studies from our lab have reported certain novel structural genes like *CLPKSII* with a putative role in biosynthesis (Deepa *et al.*, 2017). The expression of these structural genes is regulated at the transcriptional level by TFs. TFs are sequence-specific DNA-binding proteins that interact with the regulatory regions of the target genes and control transcriptional initiation rate (Vom Endt *et al.*, 2002). These regulators bind to homologous *cis*-elements situated in promoters and regulate the expression of biosynthetic pathway genes or interact with other TFs to form a complex (Yang *et al.*, 2012, Zhao *et al.*, 2019). Several families of TFs have been described as

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regulators of plant secondary metabolism that include bHLH, WD40, MYB, WRKY, bZIP, NAC *etc.* (Zhao *et al.*, 2013; Saga *et al.*, 2012; Xu *et al.*, 2004; Wang *et al.*, 2015). Biosynthesis of phenylpropanoids is generally controlled by a gene regulatory network involving MYB transcription factors regulating early biosynthetic steps and a complex of MYB-bHLH-WD40 TFs regulating the late biosynthetic steps (Li *et al.*, 2014). Late biosynthetic steps are more specific to the final metabolite and are more important for pathway engineering. We have already reported MYB TFs regulating curcumin biosynthesis in an earlier study (Sheeja *et al.*, 2015); however, no information on key TFs such as bHLH and WD40 are available. Hence, in the present study, we evaluated bHLH and WD40 TFs and identified three novel TFs that negatively regulate curcumin biosynthesis, which may lead to unravelling the regulatory mechanism.

Materials and methods

Plant material

Rhizomes were collected after 120 days of planting from high-curcumin turmeric- IISR Prathibha, low curcumin turmeric- Accession No. 200 and a closely related species that is practically devoid of curcumin- *C. aromatica* from the Experimental Farm, Chelavoor of ICAR-Indian Institute of Spices Research (ICAR-IISR), Kozhikode, Kerala State, India (Table 1).

Screening of *Curcuma* transcriptome for different transcription factor unigenes

Homologous transcripts of different types of TFs like bHLH, WD40, NAC, WRKY, bZIP with fold change >3 was retrieved from the text of annotated genes within transcriptome data of *C. longa* (high curcumin) and *C. aromatica* (practically devoid of curcumin) (Sheeja *et al.*, 2015) (Table 2). Gene ontology (GO) terms were

retrieved for each unigene using the default settings of Blast 2GO software (Conesa and Gotz, 2008).

Quantitative real-time PCR

Total RNA was isolated as per Deepa *et al.* (2014), and three biological replicates were pooled for each sample and were digested using an on-column DNase I digestion set. The quality and quantity of RNA were checked by agarose gel electrophoresis (1.2%) and Biophotometer plus (Eppendorf, Germany). The RNA samples showed two discrete bands of 28S and 18S rRNA on agarose gel and A260/280 ratio between 1.8 and 2.0. About 250 ng of total RNA was used for first-strand cDNA synthesis using Superscript III reverse transcriptase (Invitrogen) and Oligo-(dT)18 primer in a total volume of 20 μ L. Gene expression profiling was performed on Rotor-Gene Q (Qiagen) using the QuantiFast SYBR Green PCR kit (Qiagen). The reaction mixture comprised of 10 μ L of 2X SYBR Green, 10 pmol each of gene-specific primers and 100 ng of cDNA in a final volume of 20 μ L. qRT-PCR amplification was performed under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 10s and 60°C for 30 sec. A melt curve program of 65-99°C was included to check the specificity of qRT-PCR products. Three technical replicates per reaction were performed, and the mean Ct value was used for the analysis. A reverse transcription negative control (without reverse transcriptase) and a non-template negative control were included to confirm the absence of genomic DNA and check non-specific amplification. The primers for 20 TFs were designed based on previous transcriptome data (Sheeja *et al.*, 2015) using Primer Quest (www.idtdna.com/primerquest/home/index); the details of the primers are listed in Table 2. Candidate reference genes *viz.*, *EFl α* (FP-GCTGACTGTGCTGTTCTCATTAT and RP-CTCGTGTCTGTCCATCCTTTGAA) and

Table 1. Details of *Curcuma* species chosen for the study and curcumin contents

Curcuma species	Source	Curcumin content (%)
IISR Prathibha	ICAR-IISR germplasm	5.37 (high)
Accession No. 200	ICAR-IISR germplasm	3.54 (low)
<i>C. aromatica</i>	ICAR-IISR germplasm	0.12 (very low)

Table 2. Gene ontology analysis and qRT-PCR primers of TFs showing differential expression in Transcriptome

Sl. No.	Contig ID	Fold change	Sequence description	Blast per. identity	Blast top hit description	GO names	Forward primer (5' -3')	Reverse primer (5' -3')
1.	647693	10.24	bHLH transcription factor [<i>Lilium</i> hybrid division I]	69.42%	<i>Musa acuminata</i> subsp. malaccensis transcription factor EGL1-like mRNA	XM_009385819.2	CTTGCTAAT GGAAGGAA CCAATG	CGGAGAATC TACTGAA GCAATG
2.	587097	8.55	Putative WRKY transcription factor [<i>Musa balbistiana</i>]	81.98%	<i>Musa acuminata</i> subsp. malaccensis WRKY transcription factor WRKY51, transcript variant X2, mRNA	XM_009420281.2	TCGTATCCA GCTCTCCA	TCCGATTCA GGACGGAGAT
3.	603014	6.29	bHLH transcription factor-like protein [<i>Musa x paradisiaca</i>]	80.13%	<i>Musa acuminata</i> subsp. malaccensis transcription factor EGL1-like, mRNA	XM_009385819.2	CTACGATGG TTATCTGTGG AGAAA	CCTTTGTC GACTGTAGGTG
4.	644375	6.19	transcription factor bHLH149-like [<i>Solanum lycopersicum</i>]	78.95%	<i>Musa acuminata</i> subsp. malaccensis transcription factor bHLH149-like, mRNA	XM_009385663.2	ATCCTCCTCT CCGGCAA	ACCCTCGTCTT CTTCTCCA
5.	659408	5.82	transcription factor WRKY71 [Musa ABB Group]	85.44%	<i>Musa acuminata</i> subsp. malaccensis WRKY transcription factor WRKY71-like, mRNA	XM_009393878.2	CTTGTCACTTT CTGGCCATAAT	CCTACTCTCCA AGTTCTACGTC
6.	668415	5.26	transcription factor bHLH30-like [<i>Fragaria vesca</i> subsp. vesca]	84.62%	<i>Ananas comosus</i> transcription factor bHLH30-like, mRNA	XM_020248645.1	CGCATCAACG GCCATCT	CCGCTTCAG CTCCTTCAC
7.	647385	5.01	NAC transcription factor ONAC010-like [<i>Setaria italica</i>]	81.60%	<i>Musa acuminata</i> subsp. malaccensis NAC domain-containing protein 35-mRNA	XM_009383787.1	GATTGGGTCAT GAACGAGTACC	CAGCGATGTAG CTTTCCTGTAT
8.	677063	5.23	F-box and wd40 domain protein, putative	78.36%	<i>Musa acuminata</i> subsp. malaccensis uncharacterized WD repeat-containing protein alr3466, transcript variant X1, mRNA	XM_009418754.2	GCTTAAGCCTC GACGAGAAG	ATGGACTCGA GGCACTTAGA
9.	510280	5.12	NAC domain protein [<i>Cymbidium</i> hybrid cultivar]	86.14%	<i>Musa acuminata</i> subsp. malaccensis NAC transcription factor 29-like, mRNA	XM_009412202.2	CGCCGACGTC GACATATAC	GGTGAAGAAGT ACCACCTCTCG
10.	647385	5.01	NAC transcription factor ONAC010-like [<i>Setaria italica</i>]	67.24%	<i>Musa acuminata</i> subsp. malaccensis NAC domain-containing protein 35, mRNA	XM_009383787.1	GATTGGGTCAT GAACGAGTACC	CAGCGATGTAG CTTTCCTGTAT

11.	663323	4.45	NAC domain-containing protein 29-like	77.37%	<i>Musa acuminata</i> subsp. malaccensis NAC domain-containing protein 83-like, mRNA	XM_009412290.2	CACAGCTTGAG GAAGAGAAAAG	AGAAAGAGGACA GGTGGAGTAG
12.	609334	10.08	WRKY31 (Fragment)	89.01%	<i>Musa acuminata</i> subsp. malaccensis WRKY transcription factor WRKY51, transcript variant X2, mRNA	XM_009420281.2	GAGCTACTGGTA AAGGAACTGG	CAGATTAAGACC TTCGTGGAGAA
13.	489876	3.8	Putative WRKY transcription factor 39 [<i>Aegilopstauschii</i>]	84.77%	<i>Musa acuminata</i> subsp. malaccensis probable WRKY transcription factor 21, transcript variant X4, mRNA	XM_009415648.2	CCGGAGGGATAT CAGATGATTTAT	AAATGGCAAA GTGCTACTACTG
14.	658926	5.06	WRKY transcription factor 54 [<i>Jatropha curcas</i>]	79.80%	<i>Musa acuminata</i> subsp. malaccensis probable WRKY transcription factor 41, mRNA	XM_009405246.2	TGCGATAGGTA GCATCTGAAAATA	GGAAGAGCG TGAGGAAAATG
15.	609452	15.59	bHLH transcription factor 1 [<i>Lilium hybrid division I</i>]	74.02%	<i>Nelumbo nucifera</i> transcription factor GLABRA 3-like, transcript variant X2, mRNA	XM_010243597.2	AGATATCACCAAT GGCAGGAAAG	CGATAGGGCCTT CITTTGGATAG
16.	632760	5.15	transcription factor bHLH150-like [<i>Setaria italica</i>]	76.46%	<i>Musa acuminata</i> subsp. malaccensis transcription factor bHLH149-like, mRNA	XM_009411209.2	GCGTTCGCTCA CCTTCTT	GCTGCAAAGTT CCTCCTCAAA
17.	660094	6.49	WRKY transcription factor 18 [<i>Oryza sativa</i> (japonica cultivar-group)]	73.02%	<i>Panicum hallii</i> WRKY transcription factor 55-like, mRNA	XM_025950044.1	CTACGATGGTTA TCTGTGGAGAAA	CCTTTGTCCGAT CTGTAGGGTG
18.	651973	4.84	putative bZIP transcription factor superfamily protein [<i>Zea mays</i>]	79.59%	<i>Durio zibethinus</i> bZIP transcription factor 18-like, mRNA	XM_022891899.1	ACATGATGTAAC CTCCCAITAGAC	TCAGATGCATGA GGCAGCTAAAT
19.	575876	4.47	Transcription factor HY5 (Protein LONG HYPOCOTYL 5) (bZIP transcription factor 56) (AtbZIP56)	86.44%	<i>Musa acuminata</i> AAA Group long hypocotyl 5 (HY5) gene, complete cds	MN394629.1	AGACTGAGCGG TGGACT	TGGAAGGCAAA GAGGGAATG
20.	664145	4.78	Transcription factor bHLH61 isoform 1 [<i>Theobroma cacao</i>]	70.14%	<i>Camellia sinensis</i> transcription factor bHLH93-like, transcript variant X1, mRNA	XM_028267206.1	GTTCGAGGAGT TGGGCTCTC	CCITGGGCTTCT CITCCAATA

Ubiquitin (FP-GCACTCTCGCTGACTACAAC and RP- GGCTTGGTGTAGGTCTTCTTC) were used as per Deepa *et al.* (2017). Standard curves were generated for each gene with serial dilutions of pooled cDNAs (10^{-1} to 10^{-6}) for calculating qRT-PCR efficiency. The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) and transformed to a log 2 scale.

Estimation of curcumin content

Curcumin content of rhizome samples was analysed spectrophotometrically at 430 nm following the American Spice Trade Association (1968) procedure (Table 1).

Results and discussion

Identification of TFs from *Curcuma* transcriptome

Transcriptome analysis is widely reported as an effective method for identifying regulatory molecules like TFs in many studies involving phenylpropanoids (Zong *et al.*, 2019; Song *et al.*, 2019; Todd *et al.*, 2010). Aligning the annotated transcripts of *C. longa* and *C. aromatica* to the AGRIS database resulted in the identification of unigenes belonging to 39 transcription factor families in *C. longa* and 36 transcription factor families in *C. aromatica*, which included bHLH, MYB, WRKY, MADS, NAC, bZIP, ARR-B, C2H2 and AP2-EREBP TFs (Sheeja *et al.*, 2015). Among these, seven bHLH (contig ID nos. 647693, 603014, 644375, 644375, 668415, 609452, 632760), six WRKY (contig ID nos. 587097, 659408, 658926, 660094, 609334, 489876), three NAC (contig ID nos. 647385, 510280, 663323), two bZIP (contig ID nos. 651973, 575876) and one WD40 (contig ID no. 677063) showed maximum fold change *vis-a-vis* curcumin levels. Several families of TFs have been described to be regulators of plant secondary metabolism, which include bHLH (Hong *et al.*, 2012; Shoji *et al.*, 2011; Zhang *et al.*, 2011; Todd *et al.*, 2010), WD40 (Li *et al.*, 2014), MYB (Teng *et al.*, 2005; Gonzalez *et al.*, 2008; Yamagishi *et al.*, 2010; Butelli *et al.*, 2012; Xu *et al.*, 2017), WRKY, (Suttipanta *et al.*, 2011; Ma *et al.*, 2009), bZIP, (Yu *et al.*, 2012), NAC (Saga *et al.*, 2012) *etc.*

Biosynthesis of phenylpropanoid derivatives like flavonoids is generally controlled by MYB TFs and MYB-bHLH-WD40 (MBW) complexes (Schaart *et al.*, 2013; Li *et al.*, 2014; Xu *et al.*, 2015).

qRT-PCR expression patterns of TFs

qRT-PCR analysis is a common strategy to validate the RNA-Seq data. Twenty shortlisted TFs from transcriptome analysis were further evaluated by qRT-PCR with IISR Prathibha as control. Among these contig IDs 603014 and 668415 (bHLH TFs) and contig ID 677063 (WD40 TF) showed the highest relative fold change with respect to curcumin (low *vs* high) (Fig. 1). The expression of *CIPKS11*, a bait gene that is found to be upregulated under high curcumin conditions (Deepa *et al.*, 2017), was high in IISR Prathibha and down-regulated in low curcumin accessions of *C. aromatica* and Accession No. 200. bHLH TFs (Contig IDs 603014 and 668415) and WD40 TF (contig ID 677063) showed a negative correlation with respect to the expression of *CIPKS11* (Fig. 1). If one or more genes in a biosynthetic pathway for a particular metabolite is identified, these genes may be used as baits to identify other novel candidates involved in the pathway through a co-expression analysis (Medema and Osbourn, 2016). Several studies have reported that bHLH members play a central role in transcriptional regulation of biosynthetic pathways by forming a complex with WD40 and MYB TF proteins (Zhao *et al.*, 2019). In flavonoid biosynthesis, additional regulators which interact with bHLHs or R2R3-MYBs to organize or disrupt the formation of the MBW complex, leading to enhanced or compromised flavonoid production, have been reported (Li *et al.*, 2014). The qRT-PCR trends were in congruence with the fold change values in RNA-seq Data. Thus, we have identified three novel TFs belonging to the bHLH and WD40 classes to add up to the several MYB TFs identified in our earlier studies (Sheeja *et al.*, 2015). Blastn search indicated the unique status of all three TFs based on the per cent identity (Table 2). These novel TFs, in conjunction with the already identified MYB TFs will be instrumental in future studies to confirm the mechanism of the molecular regulation of curcumin biosynthesis.

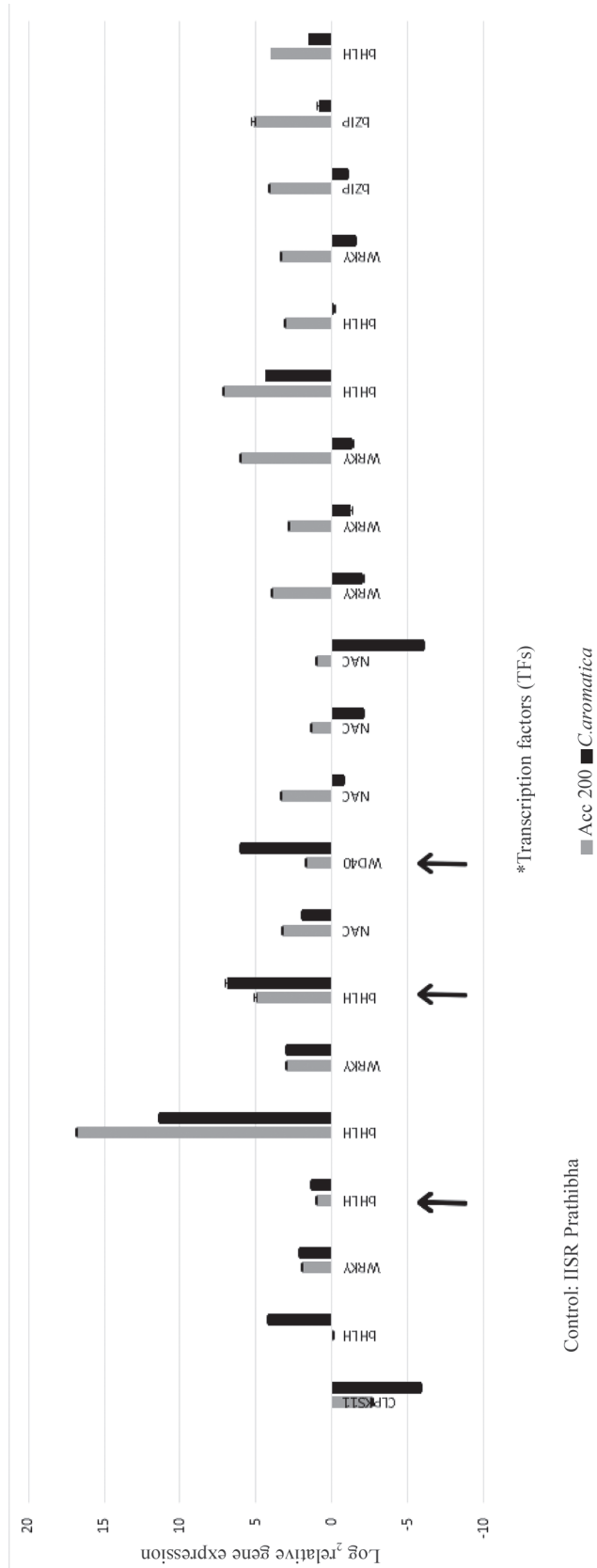


Fig. 1. Mean normalized expression of TFs mined from transcriptome. Error bars are standard errors of the mean from three technical replicates. *TFs are in the same order as in Table 2, the arrow represents the novel candidate TFs identified by qRT-PCR.

Conclusion

Curcumin is a phenylpropanoid derivative, and its biosynthesis is regulated by several classes of TFs, with bHLH, WD40 and MYB TFs being the most important. In the present study, we have identified 20 TFs through comparative transcriptome analysis. Among these, real-time expression patterns of two TFs belonging to bHLH and one belonging to WD40 categories indicated the highest differential fold change between high and low curcumin accessions and a negative correlation with curcumin content. Their expression was also negatively correlated with a bait gene *CLPKS II* in both transcriptome analysis and qRT-PCR. These novel TFs may help understand the molecular regulation of biosynthesis of curcumin, which is essential for developing high curcumin lines in turmeric.

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