



Comparative gene expression study between two turmeric (*Curcuma longa* L.) cultivars

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Abstract

Two turmeric (*Curcuma longa* L.) cultivars differing in curcumin content *viz* GNT-2 (4.6 % curcumin) and NDH-98 (1.6% curcumin) were selected for comparative gene expression study in association with total curcumin contents. Sampling was done at six months after planting in open field condition. Differential gene expression patterns were observed between two cultivars by reverse transcription-quantitative real time polymerase chain reaction (RT-qPCR), and total curcumin contents were quantified using high performance liquid chromatography (HPLC). Low curcumin yielding cultivar, NDH-98, exhibited higher expression of *DCS* and *CURS3* whereas lower expression of *CURS1* and *CURS2*. However, opposite pattern was observed in a high curcumin yielding cultivar, GNT-2, where *DCS* and *CURS3* expressions were lower but *CURS1* and *CURS2* expressions were higher. *CURS3* showed similar expression between both cultivars. *CURS1* and *CURS2* expression patterns showed more closer association than *DCS* and *CURS3* gene expression patterns with each other. Differential gene expression patterns could be predictively associated with differential curcuminoids concentrations in turmeric cultivars.

Keywords: curcumin, curcumin synthase, gene expression, turmeric

Introduction

Curcuma longa L. ($2n=3x=63$), a rhizomatous, herbaceous and perennial plant belonging to the Zingiberaceae family, is a native to Southeast Asia and widely cultivated in the tropical and subtropical regions of the world (Sheeja *et al.* 2015). Turmeric has very long history of medicinal, culinary and religious use (Prasad & Aggarwal 2011; Ravindran *et al.* 2007). *Curcuma*

contains 3–5% curcuminoids with major portion of bioactive ingredient, curcumin (Agrawal & Mishra 2010). Curcumin from this ‘golden spice’ crop is proven for its wide range of agricultural importance (Chan *et al.* 2014), pharmacological activities, cosmetic industrial value and cultural importance (Gupta *et al.* 2013; Krup *et al.* 2013). Curcumin, demethoxycurcumin and bisdemethoxycurcumin, also known as curcuminoids, are the most important bioactive

constituents of turmeric (Esatbeyoglu *et al.* 2012; Sandur *et al.* 2007). At present, traditionally known curcumin has emerged as a modern biological regulator “curcuminoids”. Agro-climatic and soil environmental variation are also influencing the curcumin synthase gene expression which is correlated with curcumin yield in turmeric cultivars (Sandeep *et al.* 2017). Novel gene identification, gene expression analysis and validations are possible with the advent of quantitative real-time polymerase chain reaction (qPCR) and RNA sequencing (RNA-Seq) methods (Li *et al.* 2015). Genomic and data mining-based studies in turmeric have identified major multiple curcumin synthase enzyme genes involved in curcuminoid biosynthesis pathway *viz* diketide-CoA synthase (*DCS*), curcumin synthase 1 (*CURS1*), curcumin synthase 2 (*CURS2*) and curcumin synthase 3 (*CURS3*) (Katsuyama *et al.* 2009a; Katsuyama *et al.* 2009b). Authors also reported that expression levels of the multiple curcumin synthases are capable of curcuminoid synthesis which might influence the composition of curcuminoids in different cultivars of turmeric. This study was aimed to describe the differential gene expressions of multiple curcumin synthases in two different cultivars with different curcumin contents which might be useful to predict association of multiple curcumin synthases with different curcumin or curcuminoids concentrations in turmeric.

Materials and methods

Handling experimental materials

To prevent RNA degradation from RNases, disposable plastic wares, glass wares, metal wares, mortar and pestle were treated with DEPC water (0.1%) overnight using Millipore (Merk) water and autoclaved for 20 minutes at 120°C. The plastic wares were dry sterilized in laminar flow with partial treatment of UV light for 30 minutes to further ensure sterile-nuclease free condition. Two turmeric cultivars, GNT-2 (4.6% curcumin, 28.7 t ha⁻¹ rhizome yield), and NDH-98 (1.6% curcumin, 33.5 t ha⁻¹ rhizome yield) grown in open field condition in research field of Department of Genetics and Plant Breeding, NMCA, Navsari Agricultural University (NAU), Navsari, India were selected for the studies. Three biological samples per cultivar were randomly selected for RNA isolation from six months old

plant at which all the aerial parts started senescing and curcumin synthesis is assumed to be stopped afterwards *i.e.* initial dormancy stage of rhizome. Rhizome fingers were excised from mother rhizome with sterile knife and washed, packed in to plastic vial along with identification number and immediately dipped in liquid nitrogen (-196 °C) container before RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated using RNeasy® Plant Mini Kit (Qiagen, Germany) following the manufacturer’s protocol. The integrity and size distribution of total RNA purified with RNeasy Kits was checked by 1.2% denaturing agarose gel electrophoresis and ethidium bromide staining visualizing two sharp bands of 28S rRNA to 18S rRNA in 2:1 ratio. The nanodrop results showed the acceptable purity of RNA isolated in each biological sample (A_{260}/A_{280} of 1.6 to 1.87 with nucleic acid concentration of 30-93 ng/μl). The isolated RNA was stored immediately at -20 °C in nuclease free water for further application. cDNA was synthesized by using Verso cDNA synthesis kit (Thermo Scientific, USA) following manufacturer’s protocol.

Primer screening through gradient PCR

After cDNA synthesis, the samples were used for gradient PCR for each pair of the gene specific forward and reverse primers along with reference genes *Actin*, (Table 1) to find out their annealing temperature and size range of PCR products in gradient PCR thermocycler (Life Sciences instrument). The best annealing temperature of qPCR primers were found to be ranging from 58-60 °C. Top Taq Master Kit (Qiagen, Germany) was used for gradient PCR following manufacturer’s protocol.

Quantitative real-time PCR (qPCR)

cDNA samples were 10-fold diluted using molecular biology grade water before using as a template in qPCR reactions. The efficiency of the reaction after dilution was confirmed by taking 1μL cDNA template from each 10-fold diluted sample for normal PCR in My Cycler PCR instrument (Bio-Rad Laboratories instrument) using Top Taq Master Mix (Qiagen, Germany) using manufacturer’s protocol. 0.2iM each of

Table 1. Gene specific primers used in standard PCR and qPCR assay

Gene	Primer sequence (5' to 3')	Primer length	Reference sequence	BLAST product size in turmeric/NCBI	Observed size in 2% agarose gel	References
<i>DCS</i> (forward)	GTGCTGTTTCATCCTGGACGAG	21bp	AB495006.1	90bp	92-94bp	(Katsuyama <i>et al.</i> 2009a)
<i>DCS</i> (reverse)	CAACAGCACGCCCCAGTCGA	20bp				
<i>CURS1</i> (forward)	CATCATTGACGCCATCGAAGC	21bp	AB495007.1	125bp	124-125bp	
<i>CURS1</i> (reverse)	TCAGCTCATCCATCACGAAGTACAC	25bp				
<i>CURS2</i> (forward)	TCGGGATCAAGGACTGGAACAAC	23bp	AB506762.1	141bp	141-142bp	(Katsuyama <i>et al.</i> 2009b)
<i>CURS2</i> (reverse)	TGTTGCCGAACCTCGGAGAAGAC	22bp				
<i>CURS3</i> (forward)	TGGAGCCCTCCTTCGACGACC	21bp	AB506763.1		95-97bp	
<i>CURS3</i> (reverse)	CCCATTCCTTGATCGCCTTTTCC	23bp				
Actin (forward)	GGATATGCTCTTCCTCATGCT	21bp	CP002686.1 AK118354.1 AY087740.1	113bp	115-116bp	(Chan <i>et al.</i> 2016)
Actin (reverse)	TCTGCTGTGGTGGTGAATGA	20bp				

[details of these primer pairs can be found in NCBI Primer Blast tool specificity check of primers against *Curcuma longa* cDNA sequences for *DCS* and *CURS*(s) genes.]

forward and reverse gene specific primers in 20 μ L reaction were used under following conditions: one cycle of initial denaturation at 94 °C for 3 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 1 minute, and one cycle of final extension at 72 °C for 10 minutes. 1.2% agarose gel electrophoresis showed the specific single band of all primers and no bands were observed in NTC (no template control) and NEC (no reverse transcriptase enzyme control) with some exception of primer dimers of less than 50 bps size in some reactions. This further confirmed the purity and abundant template quantity of cDNA synthesized in the diluted cDNA.

We selected three biological samples (replicates) and two technical samples (replicates) of each

turmeric cultivar. Single 96-well plate was set up for all the target genes as well as reference genes. qPCR was carried out in Bio-Rad CFX96 instrument using DyNAmo™ Flash SYBR™ Green qPCR Kit (Thermo Scientific, USA) using 0.5 μ M of each gene specific forward and reverse primers (Table 1) in 20 μ L final reaction volume. The reaction protocol and plate set up were carried out using Bio-Rad CFX Manager™ software. The qPCR conditions were as follows: one cycle of initial denaturation at 95 °C for 7 minutes, followed by 40 cycles of initial denaturation at 95 °C for 10 seconds, annealing/extension at 60 °C for 30 seconds and final melting curve analysis of 65 °C to 95 °C was used to check the specificity of the products according to the manufacturer's protocol (Bio-Rad CFX96 Instrument).

qPCR statistical analysis

Negative controls were also included to detect any false positives during reverse transcription and qPCR. All the generated data along with C_q values were subjected to CFX manager (v.3.1) software package which includes mathematical models for relative quantification, gene expression and normalization of qPCR data as described by Pfaffl (2001) and Vandesompele *et al.* (2002).

Results and discussion

Comparative gene expressions in GNT-2 and NDH-98

Relative normalized gene expressions were estimated in CFX manager software (Table 2) which described that *CURS1* (1.57 fold, $P=0.17$) and *CURS2* (1.46 fold, $P=0.22$) had higher fold expression in GNT-2 as compared to NDH98. However, NDH-98 exhibited higher expression of *DCS* (2.82 fold, $P=0.07$) as compared to GNT-2 (Fig. 1 and Fig. 2). This study at the initial dormancy stage of turmeric rhizome (six months from planting) described the regulated behavior of multiple curcumin synthases *viz* *DCS*, *CURS1*, and *CURS2* in turmeric rhizomes of two different cultivars under study. *CURS3* expression was almost similar between cultivars.

Gene expression and curcuminoid biosynthesis

NDH-98, a low curcumin cultivar (1.6%

curcumin), exhibited higher expression of *DCS* and *CURS3* whereas lower expression of *CURS1* and *CURS2*. However, in GNT-2, a high curcumin cultivar (4.6% curcumin), *DCS* and *CURS3* expressions were lower but *CURS1* and *CURS2* expressions were higher, at six months after planting (Fig. 1 and Table 3). However, NDH-98 has almost 5 tons/ha higher yield than GNT-2 irrespective of their curcumin content suggesting a significant difference in curcuminoid biosynthesis in rhizomes of two turmeric cultivars. *CURS1* and *CURS2* expression patterns showed more closer association than *DCS* and *CURS3* gene expression patterns in both the cultivars (Fig. 2). The results indicated that differential gene expression regulation of multiple curcumin synthases occurs in rhizome at this stage of growth. Since gene expression showed tissue specific and time specific variations (Behar *et al.* 2016), differential gene expression patterns may be observed at different stages of growth. However, in this study, *CURS3* expression was almost similar between cultivars describing the significant role of *CURS3* in curcuminoid biosynthesis. It was also found that expression levels of the multiple curcumin synthases are capable of curcuminoid synthesis with different substrate specificities which might influence the composition of curcuminoids in the turmeric and in different cultivars (Katsuyama *et al.* 2009a; Katsuyama *et al.* 2009b) indicating strong association with curcuminoids. The

Table 2 Gene expression and C_q for NDH-98 and GNT-2

Target	Biological set	Expression	Expression SD	Expression SEM	Mean C_q	C_q SD	C_q SEM
Actin	GNT-2				26.8064	0.8164	0.3651
Actin	NDH-98				27.5382	0.839	0.3425
<i>CURS1</i>	GNT-2	1.0000	0.9275	0.3279	29.5452	1.0602	0.3748
<i>CURS1</i>	NDH-98	0.6363	0.479	0.1693	30.9292	0.6895	0.2438
<i>CURS2</i>	GNT-2	1.0000	1.061	0.3537	30.8575	1.2948	0.4316
<i>CURS2</i>	NDH-98	0.6826	0.5131	0.194	32.1401	0.6872	0.2597
<i>CURS3</i>	GNT-2	1.0000	1.1175	0.3725	29.8096	1.3903	0.4634
<i>CURS3</i>	NDH-98	1.0678	0.7898	0.2633	30.4468	0.6593	0.2198
<i>DCS</i>	GNT-2	0.5892	0.5816	0.1939	29.4509	1.1669	0.389
<i>DCS</i>	NDH-98	1.6607	2.6871	1.0156	28.6878	2.1783	0.8233

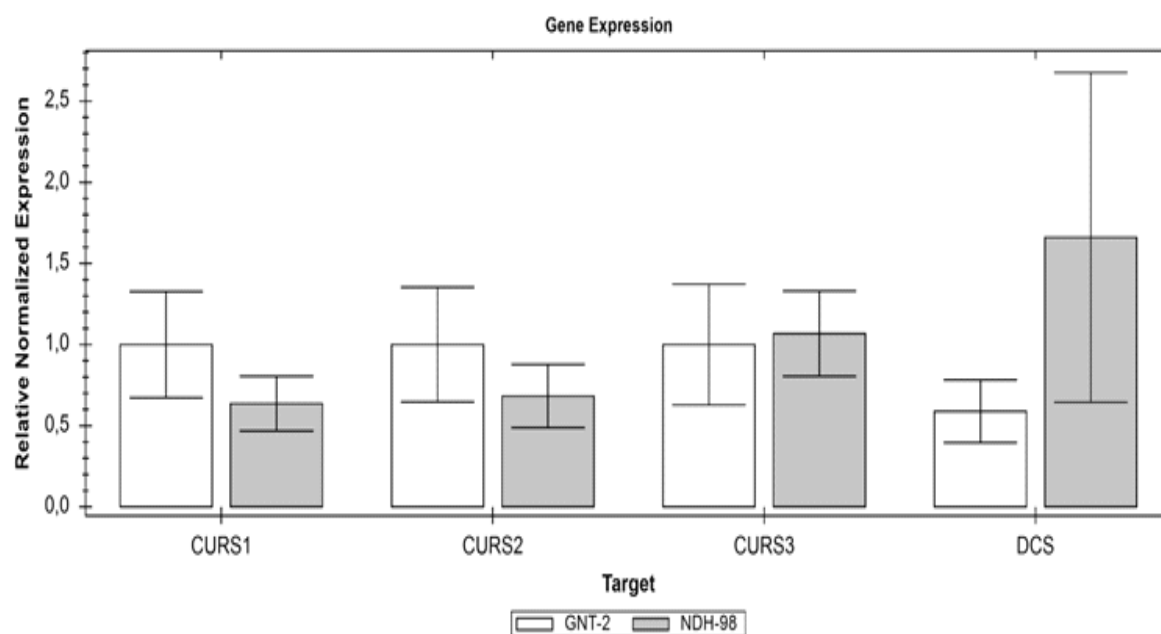


Fig. 1. Relative normalized gene expression between GNT-2 (4.6% curcumin) and NDH-98 (1.6% curcumin)

[*CURS1* and *CURS2* have higher fold expression in GNT-2 as compared to NDH98 whereas *DCS* depicted higher fold expression in NDH-98 as compared to GNT-2. *CURS3* showed similar expression between cultivars.]

expression profiles of *CURS1* are closely related to the *CURS2* expression profiles whereas that of *DCS* are closely related with *CURS3* expression profiles (Fig. 2). The gene expressions were normalized to the reference gene *Actin*. Rhizome samples were taken from turmeric cultivars at

six months after planting. At this stage of growth, plants were at the final stage of senescence i.e. initial dormancy stage. Based on the metabolic profiling study and gene expression analysis, Li *et al.* (2015) also reported that the difference in the contents of curcuminoids among

Table 3 Comparative gene regulation between GNT-2 and NDH-98

S.N.	Target	Biological set	Regulation	P-Value	Exceeds p-value threshold	Compared to regulation threshold	Control
1	Actin	NDH-98		0.9621	Yes	No change	GNT-2
2	CURS1	NDH-98	-1.5716	0.1772	Yes	No change	GNT-2
3	CURS2	NDH-98	-1.4646	0.2252	Yes	No change	GNT-2
4	CURS3	NDH-98	1.0678	0.5092	Yes	No change	GNT-2
5	DCS	NDH-98	2.8187	0.0761	Yes	Up regulated	GNT-2
6	Actin	GNT-2		0.9621	Yes	No change	NDH-98
7	CURS1	GNT-2	1.5716	0.1772	Yes	No change	NDH-98
8	CURS2	GNT-2	1.4646	0.2252	Yes	No change	NDH-98
9	CURS3	GNT-2	-1.0678	0.5092	Yes	No change	NDH-98
10	DCS	GNT-2	-2.8187	0.0761	Yes	Down regulated	NDH-98

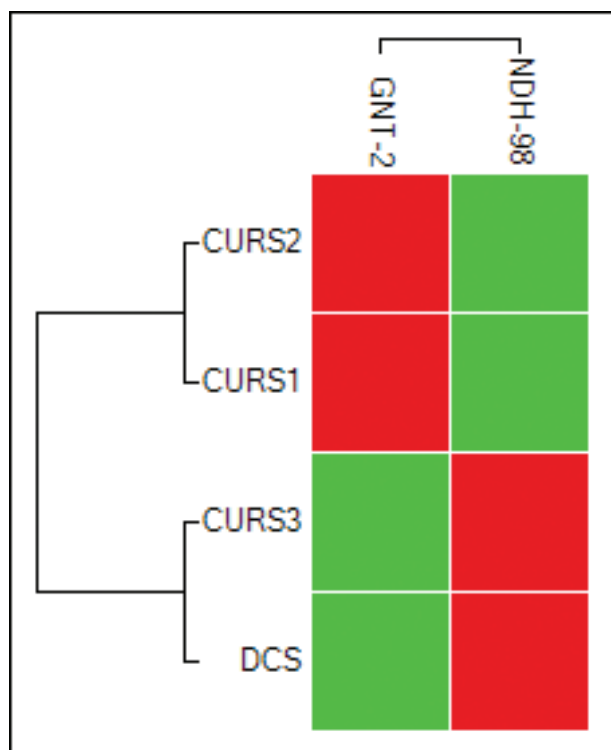


Fig. 2. Cluster diagram showing differential expression of multiple curcumin synthases and association among expression profiles [Dark red colored box represents upregulation of genes whereas green colored box represents downregulation of genes. CURS1 and CURS2 are upregulated whereas DCS and CURS3 are downregulated in GNT-2. However, CURS1 and CURS2 were downregulated whereas DCS and CURS3 genes were upregulated in NDH-98. CURS1 and CURS2 expression pattern showed more closer association than DCS and CURS3 gene expression pattern. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

the species, as measured by gas chromatography–mass spectrometry, could be explained by the changes in the expression of genes encoding diketide-CoA synthase, and curcumin synthase in curcuminoid biosynthesis pathway. They also reported that higher curcumin containing cultivars showed lower expression of DCS whereas higher expression of CURS1 and CURS2, and *vice versa* when similar stage of growth was chosen for the study as in this experiment. Expression levels of CURS3 were also found low in four different curcumin

containing cultivars as reported by Li *et al.* (2015) but were similar between leaves and rhizome samples in turmeric (Katsuyama *et al.* 2009b). The results indicated that differential gene expression profiles of different turmeric cultivars could be predictively associated with different curcuminoid contents of respective cultivars to further elucidate the curcuminoid biosynthesis pathway in medicinally important spice crop, turmeric. Further study in this aspect may explore the association of multiple curcumin synthases gene expressions with curcuminoids synthesis in turmeric.

Two cultivars differing in curcumin content *viz* GNT-2 (4.6 % curcumin) and NDH-98 (1.6 % curcumin) were taken for comparative gene expression study in association with total curcumin contents. Differential gene expression patterns between two cultivars revealed regulated behaviour of multiple curcumin synthase between two cultivars. NDH-98 exhibited higher expression of DCS and CURS3 whereas lower expression of CURS1 and CURS2. However, in GNT-2, DCS and CURS3 expressions were lower but CURS1 and CURS2 expressions were higher, at six months after planting of rhizome. CURS3 showed similar expressions between two cultivars. CURS1 and CURS2 expression patterns showed more closer association than DCS and CURS3 gene expression patterns with each other. Differential gene expression profiles were observed between two different turmeric cultivars differing in curcumin contents.

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