Associating gene expressions with curcuminoid biosynthesis in turmeric

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Abstract
Background: Biologically important curcuminoids viz curcumin, demethoxycurcumin, and bisdemethoxycurcumin in turmeric rhizome have several health benefits. Pharmaceutical industries synthesize curcuminoids manipulating gene expressions in vitro or in vivo because of their medicinal importance. In this experiment, we studied the gene expressions involved in the curcuminoid biosynthesis pathway in association with curcuminoid yield in turmeric rhizome to study the impact of individual gene expression on curcuminoid biosynthesis.

Results: Gene expressions at the different growth stages of turmeric rhizome were association tested with respective curcuminoid contents. Gene expression patterns of diketide-CoA synthase (DCS) and multiple curcumin synthases (CURS) viz curcumin synthase 1 (CURS1), curcumin synthase 2 (CURS2), and curcumin synthase 3 (CURS3) were differentially associated with different curcuminoid contents. Genotype and growth stages showed a significant effect on the gene expressions resulting in a significant impact on curcuminoid balance in turmeric rhizome. DCS and CURS3 expression patterns were similar but distinct from CURS1 and CURS2 expression patterns in turmeric rhizome. DCS expression had a significant positive and CURS3 expression had a significant negative association with curcumin, demethoxycurcumin, bisdemethoxycurcumin, and total curcuminoid in turmeric rhizome. CURS1 expression had a negative association with curcumin whereas CURS2 expression showed a positive association with demethoxycurcumin.

Conclusions: The effects of DCS and CURS expressions are not always positive with different curcuminoid contents in turmeric rhizome. DCS expression has a positive and CURS3 expression has a negative association with curcuminoids. CURS1 and CURS2 are also associated with curcumin and demethoxycurcumin synthesis. This mechanism of co-expression of diketide-CoA synthase and multiple curcumin synthases in turmeric rhizome has a significant effect on curcuminoid balance in different turmeric cultivars. Further experiment will explore more insights; however, association-based test results from this experiment can be useful in improving curcuminoid yield in turmeric rhizome or in vitro through the application of genetic engineering and biotechnology.

Keywords: Association, Curcuminoids, Diketide-CoA synthase, Gene regulation, Multiple curcumin synthases

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Background
Turmeric (Curcuma longa L. syn. Curcuma domestica Val.) is a rhizomatous, herbaceous, and perennial plant belonging to the Zingiberaceae family which includes almost 110 species [1]. Turmeric is also known by different names in different countries such as Haldi in India and Besar in Nepal. Turmeric is widely cultivated in the tropical and subtropical regions of the world including India, China, Bangladesh, Nepal, Sri Lanka, Pakistan, Asian subcontinents, and the Caribbean and Latin American regions [2]. The use of turmeric rhizome is mentioned in the Vedic culture in Indian subcontinents and still has extensive use as a culinary spice, coloring agent, food preservative, natural dye in the food industry, cosmetics, medicine, and religious rituals [3, 4]. The first evidence of biological effects of turmeric have been attributed to its constituent curcumin, discovered and isolated almost two centuries ago [5]; however, recent studies suggest that most of the pharmaceutical properties of turmeric depend on its biologically active constituents, curcuminoids viz curcumin, demethoxycurcumin, and bisdemethoxycurcumin [6]. The turmeric rhizome contains 3–5% curcuminoids and up to 5% essential oils and resins [7] where commercially available turmeric powder is generally composed of about 77% curcumin, 17% demethoxycurcumin, and 3% bisdemethoxycurcumin [6]. Curcumin, a major curcuminoid from turmeric rhizome, is proven for its significance as anti-inflammatory, antioxidant, antimutagenic, anti-diabetic, antibacterial, antiviral, antifungal, hepatoprotective, kidney disease treatment, expectorant, anti-cancerous, treating conditions like arthritis, inflammation to Alzheimer’s disease, and influencing gene transcription and expression, cell signaling pathway, and epigenetic mechanisms [3, 8–12]. Turmeric is a minor crop in terms of its contribution towards food security; however, the curcuminoids from this spice crop are attractive targets for metabolic engineering and pharmaceutical applications [13].

Curcuminoids are biologically synthesized through a phenylpropanoid pathway in turmeric [14]; however, several studies were also carried out successfully for producing curcuminoids in vitro through an artificial curcuminoid biosynthesis pathway [13, 15, 16]. There are several other upstream section enzyme genes involved in the curcuminoid biosynthesis pathway which catalyze the synthesis of early substrates (feruloyl-CoA/p-coumaroyl-CoA) [14, 17, 18]; however, downstream section enzyme genes involved in the curcuminoid biosynthesis pathway play a major role in catalyzing the formation of curcuminoid scaffold in turmeric rhizome. Diketide-CoA synthase (DCS), a type III PKS, and multiple curcumin synthases (CURSs) viz curcumin synthase 1 (CURS1), curcumin synthase 2 (CURS2), and curcumin synthase 3 (CURS3) involved in the downstream section of the curcuminoid biosynthesis pathway in turmeric were identified and characterized where DCS synthesizes feruloyldiketide-CoA/p-coumaroyldiketide CoA and CURS1, CURS2, and CURS3 then converts diketide-CoA esters into different curcuminoid scaffolds viz curcumin, demethoxycurcumin, and bisdemethoxycurcumin [19, 20]. The authors also reported that relative expression of DCS, CURS1, and CURS2 were higher in rhizome than leaf whereas CURS3 expression was almost similar in rhizome and leaf. The gene expression studies suggested that the content of curcuminoids in turmeric cultivars depends on the expression balance of these four major enzyme genes (DCS, CURS1, CURS2, and CURS3) in turmeric rhizome in addition to the availability of the substrates for curcuminoid scaffold biosynthesis. Transcriptomics studies in turmeric have shed light on the identification and validation of multiple curcumin synthases involved in the curcuminoid biosynthetic pathway in turmeric [17, 18, 21]. More novel type III PKS genes have been identified recently, such as CLPKS9 and CLPKS10 [22], CLPKS1 and CLPKS2 [18], and CLPKS11 [23] which may have a potential role in curcuminoid scaffold biosynthesis; however, the difference in the contents of curcuminoids among the species can be explained by the changes in the expression of genes encoding diketide-CoA synthase and multiple curcumin synthases at the branching point of the curcuminoid biosynthesis pathway in turmeric [24]. The variation in curcuminoid content among the various lines and cultivars of C. longa was reported to be caused by hybridization and introgression [25], agro-climatic variation [26], genotype and environment interaction [27], and microenvironment (below soil surface near root zone) and macroenvironment (above soil surface) interaction [28]; however, the effects of tissue-specific gene expressions on curcuminoid biosynthesis are still unknown. Gene expressions of diketide-CoA synthases and multiple curcumin synthases in turmeric were also found to be influenced by tissue-specific (leaves and rhizome) and temporal (growth stages) variations within a cultivar [29]; this may have an influence on curcuminoid balance. CURS expression was found regardless of the variation in the curcumin content in different turmeric cultivars at different agroclimatic regions, and expressions were positively correlated with curcumin content in turmeric cultivars within an environment at different stages of growth [30]; however, the effect of individual DCS and CURS gene expression on curcuminoid yield is still unexplored. The first-stage study of this experiment revealed that differential DCS and CURS expressions can be predictively associated with curcuminoid content in different turmeric cultivars to study the relationship between target gene expressions and curcuminoid yield in turmeric rhizome [31]. Specific genes associated with target quantitative traits are commonly known as trait-
associated genes (TAGs) which can be defined by the robust linear regression analysis through an association test between continuous traits and mRNA expression [32]. Huber’s M-estimator-based robust regression method [33] is useful for identifying TAGs. In this experiment, we studied DCS and CURS gene expressions in association with variable curcuminoid content in turmeric rhizome at different stages of growth to find out the impact of individual gene expression on curcuminoid yield in terms of magnitude and direction using a statistical model. Gene expressions or transcript abundance was regarded as genotypic values whereas associated changes in curcuminoids were regarded as phenotypic values at different growth stages while performing the association-based test. The association-based test results from this experiment can also be useful for improving curcuminoid yield in turmeric rhizome or in vitro through the application of genetic engineering and biotechnology.

Methods
General experimental conditions
Rhizome samples of turmeric cultivars (GNT-2, Pratibha, and NDH-98) were selected randomly for gene expression study and curcuminoid analysis at three different growth stages in field conditions. The first stage was determined to be the active vegetative growth stage (SI, 4 months after planting), the second stage was at the active rhizome development stage (SII, 5 months after planting), and the third stage was at the maturity or senescence stage (SIII, 6 months after planting). Three biological samples (rhizomes) from each cultivar at three stages of growth under study were taken out of the soil for RNA isolation, isolated RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA), and gene expression was studied through RT-qPCR in the Department of Genetics and Plant Breeding and Biotechnology Laboratory at NAU, Gujarat, India, during 2017–2018. The cultivars used in this experiment were denoted as biological sets NDH-98 (N), GNT-2 (G), and Pratibha (P). From each biological sample, two technical replicates were utilized for further downstream analysis through qPCR. The gene expression results obtained from the RT-qPCR assay were further used for association tests with curcuminoid yield in turmeric rhizome. Plant sampling procedures for RNA isolation were carried out following the recommended guidelines [34]. Technical sampling procedures and RT-qPCR procedures were followed as per the MIQE guidelines for the gene expression study [35, 36].

Rhizome sampling and curcuminoid content analysis
At each stage of study viz SI, SII, and SIII, rhizome fingers were excised from the mother rhizome with a sterile knife and washed, packed into aluminum foil along with identification number, and immediately dipped into liquid nitrogen (−196 °C) container for RNA isolation, cDNA synthesis, and gene expression study in the laboratory. The remaining portions of individually sampled rhizomes were cleaned, sliced into pieces, sun-dried for 5–7 days, and made into a fine powder using the grinder for curcuminoid content analysis (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) through HPLC using individual curcuminoid standards (Sigma Aldrich) at the Food Quality Testing Laboratory (FQTL), NAU, Navsari, Gujarat. For curcuminoid analysis, 1.0 g turmeric powder of each cultivar was dissolved in 50 ml of 100% methanol separately and then mixed and vortexed for 30 min. The solution is then filtered and injected in the Thermo HPLC Surveyor instrument (Thermo Scientific) for individual curcuminoid measurement at 425-nm wavelength. Laboratory analysis was performed at room temperature (25–30 °C).

RNA isolation and cDNA synthesis
Total RNA was isolated at three different growth stages (SI, SII, and SIII) of three turmeric cultivars under study using the RNeasy® Plant Mini Kit (Qiagen, Germany) involving on-column genomic DNA digestion step of DNase I (Qiagen) treatment following the manufacturer’s protocol. The integrity and size distribution of the total RNA purified were checked by 1.2% denaturing formaldehyde agarose (FA) gel electrophoresis and staining with ethidium bromide visualizing two sharp bands of 28S rRNA to 18S RNA in a 2:1 ratio. RNA purity check and quantification were done using the Nanodrop 2000 instrument (Thermo Scientific) which showed the acceptable purity for each biological sample (A260/A280 of 1.9 to 2.1). From the total RNA, the mRNA with the poly-A tail was reverse transcribed into cDNA using oligo dT primers during the reverse transcription step. cDNA was synthesized in 20 μl reaction using 2 μl of total isolated RNA (1 μg in final solution) with 4 μl of 5X cDNA synthesis buffer (1X final), 2 μl of 2X dNTP mix (500 μM each), 1 μl of anchored oligo dT primer (for mRNA reverse transcription), 1 μl RT enhancer, 1 μl reverse enzyme mix, and water up to 20 μl. cDNA synthesis protocol involved a single 30-min cycle of polymerase enzyme activity at 42 °C followed by one additional cycle of 2 min at 95 °C for inactivation of enzyme (Verso cDNA Synthesis Kit, Thermo Scientific).

Quantification of transcript abundance through RT-qPCR assays
RT-qPCR assays at three different stages were carried out along with gene-specific and reference gene primers (Additional file 1: Table S2) which were validated in turmeric RNA samples. All the qPCR assays of four target
genes as well as reference genes at each stage were included in a single 96-well plate (Thermo Scientific) for minimizing error while quantifying the abundance of cDNA in technical replicates of each biological sample. A standard qPCR protocol and plate setup were performed on CFX96 real-time PCR thermocycler using the CFX Manager software (Bio-Rad). For finding out transcript abundance in different turmeric samples, qPCR assay comprised 10μl of 2X PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 0.5μM of each gene-specific forward and reverse primers along with reference gene Actin, 2μl of 10-fold diluted cDNA as a template, and remaining quantity of nuclease-free water (HiMedia) to make a final volume of 20μl. qPCR conditions for gene expression study were as follows: heat-labile uracil-DNA glycosylase (UDG) activation at 50°C for 2 min followed by Dual-Lock™ DNA polymerase activation at 95°C for 2 min, and 40 cycles of denaturation at 95°C for 15s followed by combined anneal/extend at 95°C to 90°C with a 0.5°C increment was used according to the manufacturer’s protocol (Bio-Rad, CFX96 instrument), and the products were also confirmed on 1.2% agarose gel electrophoresis. Quantification cycle (Cq) and normalized gene expression values obtained in the CFX Manager software were used for further analysis. Negative controls (no template control (NTC)) were also included to detect any false positives during reverse transcription (RT) and qPCR assays. The data generated along with the Cq values were subjected to the CFX Manager (v.3.1, Bio-Rad) software package which includes mathematical models for estimating the relative quantification and normalization of qPCR data for gene expression analysis [37, 38]. Gene study feature in the CFX Manager software was set to a regulation threshold of 2-fold change and a value threshold of 0.05 for finding out any significant variations in the gene expressions. DCS and CURSs melt peak curves were studied for the specificity of the products, and gel electrophoresis was also performed for the validation of the qPCR products using the reference gene Actin.

Association-based test between gene expressions and curcuminoids

In this experiment, diketide-CoA synthase (DCS) and multiple curcin syntheses (CURS1, CURS2, and CURS3) or CURSs gene expression patterns were studied at three different growth stages viz SI (active vegetative stage), SII (active rhizome development stage), and SIII (maturity or senescence stage) among three turmeric cultivars significantly differing in curcuminoid (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) contents in the rhizome. The robust regression analysis was used by taking normalized gene expression values from the RT-qPCR analysis as explanatory or independent and curcuminoid contents (curcumin, demethoxycurcumin, bisdemethoxycurcumin, and total curcuminoid) obtained from HPLC analysis as a response or dependent variables for identifying genes that are more closely associated with curcuminoid biosynthesis in turmeric, also known as trait-associated genes (TAGs). A separate association-based test was also done for the total curcuminoid content in turmeric rhizome which was estimated by summing up the individual curcuminoid content in each turmeric rhizome at each growth stage. The association between curcuminoids and mRNA expressions was analyzed using Huber’s M-estimator-based ROBUSTREG procedure [39] in SAS® University Edition (SAS® Studio 3.71, SAS® Institute Inc.). The general linear regression model for the association-based test [32] using gene expression data was as follows:

\[
\text{Trait}_i \approx \beta_0 + \beta_1 \text{Expression}_{1i} + \ldots + \beta_p \text{ Covariate}_p + \epsilon_i, \quad \epsilon_i \sim (N(0, \sigma^2))
\]

where \( i \) represents the individuals; Expression, indicates the normalized gene expression value; Trait, represents each continuous trait such as curcumin, demethoxycurcumin, bisdemethoxycurcumin, and total curcuminoid; Covariate represents the covariate effects of factors other than error factors (\( \epsilon_i \)); \( \beta \) in a linear regression model is a standardized coefficient indicating the magnitude of the correlation between an independent and dependent variable; \( N \) and \( \sigma^2 \) represent the analysis of variance assumption of normal distribution and common standard deviation, respectively. Additional tests like the rho (\( \rho \)) test based on the robust F-test and \( R^2_n \) test based on the Wald-type tests were also carried out to measure the association of covariates on response variables. The SAS codes and dataset used during the data analysis are given in Additional file 1: Annex 1.

**Results**

**Curcuminoid analysis in turmeric cultivars**

Based on the high-performance liquid chromatography (HPLC) analysis, turmeric cultivars “GNT-2” and “Pratibha” were not significantly different for curcumin and total curcuminoid content whereas “NDH-98” was with relatively lower curcumin and lower total curcuminoid content than “GNT-2” and “Pratibha” (Table 1). There was no significant difference among the cultivars for demethoxycurcumin content; however, the highest bisdemethoxycurcumin was in “GNT-2” followed by “Pratibha” and below detection level in “NDH-98” turmeric rhizome. Irrespective of the curcuminoid contents, the rhizome yield of short-growth-duration cultivar “NDH-98” was the highest (33–34 t ha\(^{-1}\)) followed by long-growth-duration cultivars “GNT-2” (28–29 t ha\(^{-1}\)) and “Pratibha” (23–24 t

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Ayer et al. Journal of Genetic Engineering and Biotechnology (2020) 18:83
The results showed that the higher turmeric rhizome yield does not necessarily mean higher curcuminoids in the turmeric cultivars. The chromatograms showing individual curcuminoid detection in the HPLC system are given in Additional file 2.

### Gene expressions in turmeric rhizome at different growth stages

There were no off products in the qPCR analysis based on the melt peak curves which was further confirmed by gel electrophoresis showing a single band of PCR product, and the data was considered reliable for further analysis. The results of the qPCR analysis are given in Additional file 2. Comparing the gene expression profiles in different turmeric cultivars, **DCS** (2.94-fold) and **CURS3** (2.87-fold) expressions were upregulated at SII as compared with SI and SIII; however, **CURS1** (1.88-fold) and **CURS2** (1.05 fold) were expressed at a lower level in GNT-2. In NDH-98, **DCS** (0.88-fold) and **CURS3** (1.39-fold) expressions were lower; however, **CURS1** (2.22 fold) expression was upregulated at SII as compared with SI and SIII (Fig. 1 and Additional file 1: Table S1). Generally, the gene expressions were increasing from active vegetative stage to rhizome development stage (SI to SII) and decreasing at maturity or senescence stage (SIII) under study except that **DCS** expression was decreasing throughout the growth stages in NDH-98, **CURS2** expression was increasing throughout the growth stages in GNT-2, and **CURS3** expression was decreasing throughout the growth stages in Pratibha (Fig. 1). These patterns in each cultivar might have some significant role to play for curcuminoid yield in different turmeric cultivars.

### Pooled gene expressions in turmeric cultivars

The highest **DCS** expression was in higher curcumin yielding cultivar GNT-2 (1.55-fold) followed by Pratibha (1.04-fold) and the least in low curcumin yielding cultivar NDH-98 (0.66-fold) over the growth stages under study. **CURS1** and **CURS2** gene expressions were highest in NDH-98 (1.34-fold and 2.65-fold, respectively) followed by GNT-2 (0.92-fold and 0.74-fold, respectively) and Pratibha (0.84-fold and 0.49-fold, respectively). **CURS3** expressions were lower than **DCS** expressions in all the cultivars over growth stages with the highest expression in GNT-2 (1.29-fold) followed by NDH-98 (0.91-fold) and Pratibha (0.77-fold). Among all the genes under study, **CURS2** expressions were lower in Pratibha (0.48-fold and GNT-2 (0.74-fold), however significantly higher in NDH-98 (2.65-fold). The results of pooled gene expressions over growth stages are in Fig. 2 and Additional file 1: Table S1. The results showed that GNT-2 and Pratibha, higher curcuminoid yielding cultivars, had a higher **DCS** expression with relatively lower **CURS1**, **CURS2**, and **CURS3** expressions opposite to that

### Table 1 Curcuminoid contents in turmeric cultivars at different stages under study

| Sample ID | Curcumin (%) | Demethoxy curcumin (%) | Bisdemethoxycurcumin (%) | Total curcuminoid (%) | Rhizome yield (t ha⁻¹) | Growth duration (days) |
|-----------|--------------|------------------------|--------------------------|-----------------------|------------------------|------------------------|
| NDH-98_SI | 0.27         | 0.76                   | BDL                      | 1.03                  |                        |                        |
| NDH-98_SII| 0.10         | 0.36                   | BDL                      | 0.46                  |                        |                        |
| NDH-98_SIII| 0.04         | 0.08                   | BDL                      | 0.12                  |                        |                        |
| Average NDH-98 | 0.14b (25%) | 0.40a (75%)         | BDL                      | 0.54b (100%)          | 33–34                  | 240                    |
| GNT-2_SI | 2.31         | 0.71                   | 1.14                     | 4.16                  |                        |                        |
| GNT-2_SII| 0.76         | 0.07                   | 0.15                     | 0.98                  |                        |                        |
| GNT-2_SIII| 0.81         | 0.18                   | 0.24                     | 1.23                  |                        |                        |
| Average GNT-2 | 1.29a (61%) | 0.32a (19%)          | 0.51a (24%)             | 2.12a (100%)          | 28–29                  | 256                    |
| Pratibha_SI | 0.56         | 0.11                   | 0.14                     | 0.81                  |                        |                        |
| Pratibha_SII| 1.29         | 0.17                   | 0.14                     | 1.6                   |                        |                        |
| Pratibha_SIII| 1.33         | 0.22                   | 0.24                     | 1.79                  |                        |                        |
| Average Pratibha | 1.06a (76%) | 0.17a (12%)          | 0.17b (12%)              | 1.40ab (100%)         | 23–24                  | 256                    |

*“a” and “b” are the significance letters which indicate that values with the same letters in a column are statistically not significant from each other at \( p < 0.05. \)

NDH-98, GNT-2, and Pratibha turmeric cultivars

BDL Below the detection limit during HPLC analysis, SI 4 months after planting, SII 5 months after planting, SIII 6 months after planting.
of NDH-98, relatively lower curcuminoid yielding cultivar, which depicted lower DCS expression with relatively higher CURS1, CURS2, and CURS3 expressions (Fig. 2). There was no significant change in DCS, CURS1, CURS2, and CURS3 gene expressions between GNT-2 and Pratibha (higher curcumin and total curcuminoid yielding cultivars) at all stages of growth; however, their expressions differed in NDH-98 (lower curcumin and total curcuminoid yielding cultivar) (Table 2). DCS expression was found downregulated (−2.35- and −1.57-fold) whereas CURS2 expression was found upregulated (3.57- and 5.46-fold) in lower curcumin and lower total curcuminoid yielding cultivar (NDH-98) as compared with higher curcumin and higher total curcuminoid yielding cultivars (GNT-2 and Pratibha) and vice versa (Table 2). There was no significant change in the CURS3 expression (<2-fold change) among the cultivars.

Cluster analysis of DCS and CURSs gene expressions
Cluster analysis of the gene expressions of three turmeric cultivars at three different growth stages revealed that DCS and CURS3 expression patterns were similar but distinct from CURS1 and CURS2 expression patterns in turmeric rhizome (Fig. 3). Similar results were obtained when gene expression was studied in two turmeric cultivars, GNT-2 and NDH-98, after 6 months of planting in field conditions [31].

DCS and CURSs gene expressions associated with curcuminoid biosynthesis in turmeric
There was a significant effect of DCS, CURS1, and CURS3 gene expressions on curcumin content at $p < 0.01$. DCS had a positive linear association whereas CURS1 and CURS3 expressions had a negative linear association with curcumin. The covariate effect of CURS1 and CURS2 expressions on curcumin was also found significant at $p < 0.01$ although the correlation was significant at $p < 0.08$. Thus, DCS had a significantly positive whereas CURS1 and CURS3 expressions had a significantly negative association with curcumin yield in turmeric rhizome. Co-regulated expressions of CURS1 and
CURS2 were also found significantly associated with curcumin synthesis in turmeric rhizome (Table 3).

For demethoxycurcumin, DCS and CURS2 expressions had a significant positive association whereas CURS3 expressions had a significant negative association with demethoxycurcumin at \( p < 0.01 \). The covariate effect of CURS1 and CURS2 expressions on demethoxycurcumin was also found highly significant \( (p < 0.01) \). Thus, DCS and CURS2 expressions had significant positive associations whereas CURS3 expression had a significant negative association with demethoxycurcumin. Co-regulated expression of CURS1 and CURS2 also had a significant

### Table 2. DCS and CURSs gene regulation in turmeric rhizome over growth stages

| Target | Experimental sample | Regulation | \( p \) value | Exceeds \( p \) value threshold | Compared to regulation threshold | Control sample |
|--------|---------------------|------------|---------------|---------------------------------|---------------------------------|----------------|
| Actin  | GNT-2               | NA         | 0.790985      | Yes                             | No change                       | Pratibha        |
| CURS1  | GNT-2               | 1.09       | 0.179227      | Yes                             | No change                       | Pratibha        |
| CURS2  | GNT-2               | 1.53       | 0.192329      | Yes                             | No change                       | Pratibha        |
| CURS3  | GNT-2               | 1.66       | 0.003796      | No                              | No change                       | Pratibha        |
| DCS    | GNT-2               | 1.50       | 0.369600      | Yes                             | No change                       | Pratibha        |
| Actin  | NDH-98              | NA         | 0.496892      | Yes                             | No change                       | Pratibha        |
| CURS1  | NDH-98              | 1.59       | 0.730277      | Yes                             | No change                       | Pratibha        |
| CURS2  | NDH-98              | 5.46       | 0.000001      | No                              | Upregulated                     | Pratibha        |
| CURS3  | NDH-98              | 1.17       | 0.395052      | Yes                             | No change                       | Pratibha        |
| DCS    | NDH-98              | −1.57      | 0.044860      | No                              | No change                       | Pratibha        |
| Actin  | NDH-98              | NA         | 0.689920      | Yes                             | No change                       | GNT-2           |
| CURS1  | NDH-98              | 1.45       | 0.036328      | No                              | No change                       | GNT-2           |
| CURS2  | NDH-98              | 3.57       | 0.000027      | No                              | Upregulated                     | GNT-2           |
| CURS3  | NDH-98              | −1.43      | 0.022002      | No                              | No change                       | GNT-2           |
| DCS    | NDH-98              | −2.35      | 0.000368      | No                              | Downregulated                   | GNT-2           |

*+" or no sign in the regulation column represents upregulation (higher expression) of a gene in the current sample as compared with the control sample

*"−" sign indicates downregulation (lower expression) of a gene in the current sample as compared with the control sample

Upregulation of a “gene” in experimental sample as compared with control also indicates the downregulation of that same “gene” in the control sample as compared with experimental sample and vice versa. Gene expressions at different stages of growth viz SI, SII, and SIII for each cultivar were pooled together for comparative gene regulation study.
impact on the demethoxycurcumin content in the turmeric rhizome (Table 4).

\( DCS \) \((p < 0.09)\) depicted a positive effect whereas \( CURS3 \) \((p < 0.01)\) showed a significant negative effect on bisdemethoxycurcumin content. The covariate effects of \( CURS1 \) and \( CURS2 \) expression on bisdemethoxycurcumin were also found non-significant at \( p < 0.05 \). Thus, \( DCS \) expression had a positive effect and \( CURS3 \) expression had a significant negative effect on bisdemethoxycurcumin content in turmeric rhizome (Table 5).

Only \( DCS \) and \( CURS3 \) expressions had a significant effect \((p < 0.01)\) on the total curcuminoid content in turmeric rhizome where \( DCS \) had a significant positive linear association and \( CURS3 \) had a significant negative linear association with total curcuminoid. The covariate effect of \( CURS1 \) and \( CURS2 \) expression was found non-

**Table 3** Association-based test of \( DCS \) and multiple curcumin synthases with curcumin

| Parameter          | Class label | Main effects | Chi-square | Pr > ChiSq | \( \text{CURS1 and CURS2 as a covariate} \) | Estimate | Chi-square | Pr > ChiSq |
|--------------------|-------------|--------------|------------|-----------|------------------------------------------|----------|------------|-----------|
| Intercept          |             | 1.1091       | 486.32     | < .0001   | 1.1485                                   | 327.39   | < .0001    |           |
| Growth stage SI    |             | 0.1039       | 4.05       | 0.0442    | -0.1792                                  | 4.53     | 0.0333     |           |
| Growth stage SII   |             | 0.4701       | 57.26      | < .0001   | 0.0073                                   | 0.01     | 0.936      |           |
| Genotype GNT-2     |             | 1.0375       | 342.2      | < .0001   | 1.1168                                   | 161.13   | < .0001    |           |
| Genotype NDH-98    |             | -0.2774      | 14.84      | 0.0001    | -0.616                                   | 61.29    | < .0001    |           |
| Genotype Pratibha  |             | 0            |           |           | 0                                        | 0        |           |           |
| \( DCS \)          |             | 0.9126       | 82.3       | < .0001   | 0.6182                                   | 26.03    | < .0001    |           |
| \( CURS1 \)        |             | -0.3392      | 12.48      | 0.0004    | 0                                        | 0        |           |           |
| \( CURS2 \)        |             | -0.0033      | 0.01       | 0.9371    | 0                                        | 0        |           |           |
| \( CURS3 \)        |             | -1.3922      | 335.16     | < .0001   | -1.2216                                  | 153.92   | < .0001    |           |

Reduced parameter robust linear test for curcumin

\[ \text{Rho} \]

\[ R^2 \]

\[ Pr < \text{ChiSq} \] approximately predicts the associated probability level after the selected variable has been entered or removed.

\( Pr \) Probability, \( \text{ChiSq} \) Chi-square, \( \text{Rho} \) association or correlation, \( R^2 \) robust Wald-type association test.
Discussion

Gene expression studies can quantify the amount of functional mRNA (messenger ribonucleic acid) transcript in the experimental sample measured in terms of reverse-transcribed cDNA (complementary deoxyribonucleic acid) concentration which codes for amino acids and functional proteins. After the identification and characterization of multiple type III PKS enzyme genes involved in the curcuminoid biosynthesis pathway [19, 20], it is now possible to study the differential gene expression patterns using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) assays in addition to protein-enzyme and product assays in different tissues of turmeric. The role of DCS and CURSs for curcuminoid biosynthesis in turmeric has been described by different studies (Fig. 4); however, the

Table 4 Association-based test of DCS and multiple curcumin synthases with demethoxycurcumin

| Parameter | Class label | Main effects        | CURS1 and CURS2 as a covariate |
|-----------|-------------|---------------------|-------------------------------|
|           |             | Estimate  | Chi-square | Pr > ChiSq | Estimate  | Chi-square | Pr > ChiSq |
| Intercept |             | −0.1443  | 32.09      | < .0001    | −0.1497  | 12.45      | 0.0004     |
| Growth stage SI |           | 0.3705  | 200.58     | < .0001    | 0.3895   | 73.06      | < .0001    |
| Growth stage SII |          | 0.2073  | 43.38      | < .0001    | 0.2591   | 27.78      | < .0001    |
| Growth stage SIII |         | 0        | 0          | 0          | 0        | 0          | 0          |
| Genotype GNT-2 |           | 0.4197   | 218.18     | < .0001    | 0.4175   | 76.88      | < .0001    |
| Genotype NDH-98 |            | 0.3327   | 83.19      | < .0001    | 0.4616   | 117.48     | < .0001    |
| Genotype Pratibha |          | 0        | 0          | 0          | 0        | 0          | 0          |
| DCS |             | 0.2596   | 25.95      | < .0001    | 0.2913   | 19.72      | < .0001    |
| CURS1 |            | 0.0296   | 0.37       | 0.5431     | 0        | 0          | 0          |
| CURS2 |            | 0.0535   | 6.44       | 0.0111     | 0        | 0          | 0          |
| CURS3 |            | −0.4059  | 110.98     | < .0001    | −0.4188  | 61.76      | < .0001    |

Reduced parameter robust linear test for demethoxycurcumin

| Parameter | Class label | Main effects        | CURS1 and CURS2 as a covariate |
|-----------|-------------|---------------------|-------------------------------|
|           |             | Estimate  | Chi-square | Pr > ChiSq | Estimate  | Chi-square | Pr > ChiSq |
| Intercept |             | 0.3381    | 9.58       | 0.002      | 0.3586    | 12.19      | 0.0005     |
| Growth stage SI |           | 0.1514    | 1.82       | 0.1771     | 0.1847    | 2.8        | 0.094      |
| Growth stage SII |          | 0.1877    | 1.93       | 0.1644     | 0.0847    | 0.51       | 0.4768     |
| Growth stage SIII |         | 0        | 0          | 0          | 0        | 0          | 0          |
| Genotype GNT-2 |           | 0.5534    | 20.63      | < .0001    | 0.6544    | 32.23      | < .0001    |
| Genotype NDH-98 |            | −0.00154  | 0.01       | 0.9216     | −0.1401   | 1.85       | 0.1741     |
| Genotype Pratibha |          | 0        | 0          | 0          | 0        | 0          | 0          |
| DCS |             | 0.3727    | 2.91       | 0.0881     | 0.0342    | 0.05       | 0.8294     |
| CURS1 |            | −0.2976   | 2.03       | 0.1537     | 0        | 0          | 0          |
| CURS2 |            | 0.0424    | 0.22       | 0.6389     | 0        | 0          | 0          |
| CURS3 |            | −0.618    | 13.99      | 0.0002     | −0.4367   | 11.46      | 0.0007     |

Reduced parameter robust linear test for bisdemethoxycurcumin

| Parameter | Class label | Main effects        | CURS1 and CURS2 as a covariate |
|-----------|-------------|---------------------|-------------------------------|
|           |             | Estimate  | Chi-square | Pr > ChiSq | Estimate  | Chi-square | Pr > ChiSq |
| Intercept |             | 0.5115    | 12.68      | 0.0018     | 19.0271   | 19.03      | < .0001    |

Pr < ChiSq approximately predicts the associated probability level after the selected variable has been entered or removed

Pr Probability, ChiSq Chi-square, Rho association or correlation, R^2 robust Wald-type association test
correlation or directional association between the gene expression level and curcuminoid level in turmeric rhizome is still unfolded. In this experiment, three cultivars differing in curcuminoid contents were evaluated for DCS and CURSs gene expressions, and normalized expression values are association tested with curcuminoid contents (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) measured at three different growth stages in turmeric cultivars.

The curcuminoid analysis results showed that a higher turmeric rhizome yield does not necessarily mean higher curcuminoid contents in the turmeric cultivars. The curcuminoid contents were higher in long-growth-duration cultivar than short-growth-duration cultivar irrespective of the rhizome yield (Table 1). Differential gene expression patterns were also observed in three turmeric cultivars yielding different amounts of curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) measured at three different growth stages in turmeric cultivars.

The curcuminoid analysis results showed that a higher turmeric rhizome yield does not necessarily mean higher curcuminoid contents in the turmeric cultivars. The curcuminoid contents were higher in long-growth-duration cultivar than short-growth-duration cultivar irrespective of the rhizome yield (Table 1). Differential gene expression patterns were also observed in three turmeric cultivars yielding different amounts of curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin). In general, higher gene expressions were during active vegetative and rhizome development stage (SI–SII) of turmeric cultivars than reduced expression at maturity or senescence stage (SIII) under study except that DCS expression was decreasing in NDH-98 and CURS3 expression was decreasing in Pratibha throughout the growth stages (Fig. 1). Differential expression of CURSs genes was also reported to be positively correlated with the curcumin content in Curcuma spp. during the 3rd and 6th months (active vegetative and rhizome developmental stages); however, during the 9th month (maturity stage), the curcumin content increased and the putative CURSs gene expressions were downregulated [40]. These patterns in each cultivar might have some significant role to play for curcuminoid biosynthesis in different turmeric cultivars suggesting that the genotype and growth stages affect the DCS and CURSs expressions at different developmental stages in turmeric. It was also reported that the highest expression of multiple curcumin synthases in Curcuma caesia Roxb. was found at the peak of its vegetative stage and expression levels of CURSs decreased at the time of maturity [29]. Among the three cultivars under study, there was no significant difference in DCS, CURS1, CURS2, and CURS3 gene expressions between GNT-2 and Pratibha (higher curcumin and total curcuminoid yielding cultivars); however, their expressions differed in NDH-98 (lower curcumin and total curcuminoid yielding cultivar) (Table 2). DCS expression was downregulated whereas CURS2 expression was upregulated in lower curcumin and lower total curcuminoid yielding cultivar (NDH-98) as compared with higher curcumin and higher total curcuminoid yielding cultivar (GNT-2 and Pratibha) and vice versa (Table 2). There was no significant change in the CURS3 expression (<2-fold change) among the cultivars. CURS3 expressions were also found lower in all the turmeric species differing in curcuminoid yield [24]. These results showed that there is a significant variation in DCS and CURSs gene expressions in turmeric rhizome also affected by genotype and growth stages.

The expressions of CURSs genes were also found to be affected by soil, climate, and harvesting time; however, CURSs expressions were found positively correlated with the curcumin content at a particular agroclimatic zone [30]. The expression of curcumin synthase genes (CURSs) and subsequent curcumin production varied at the different developmental stages of the plant in

| Parameter | Class label | Main effects | CURS1 and CURS2 as a covariate |
|-----------|-------------|--------------|--------------------------------|
|           |             | Estimate     | Chi-square | Pr > ChiSq | Estimate | Chi-square | Pr > ChiSq |
| Intercept |             | 1.0927       | 45.46      | < .0001    | 1.2257   | 128.64     | < .0001    |
| Growth stage SI |             | 0.7981       | 23         | < .0001    | 0.9247   | 63.49      | < .0001    |
| Growth stage SI |             | 1.0051       | 25.2       | < .0001    | 0.8874   | 50.23      | < .0001    |
| Growth stage SI |             | 0            |            |            | 0        |            |            |
| Genotype GNT-2 |             | 2.1593       | 142.72     | < .0001    | 2.4428   | 405.65     | < .0001    |
| Genotype NDH-98 |             | 0.1852       | 0.64       | 0.4247     | −0.2492  | 5.28       | 0.0216     |
| Genotype Pratibha |             | 0            |            |            | 0        |            |            |
| DCS       |             | 1.545        | 22.71      | < .0001    | 0.5921   | 12.57      | 0.0004     |
| CURS1     |             | −0.5897      | 3.63       | 0.0567     | 0        |            |            |
| CURS2     |             | 0.0863       | 0.41       | 0.5201     | 0        |            |            |
| CURS3     |             | −2.4214      | 97.63      | < .0001    | −1.838   | 183.38     | < .0001    |

Reduced parameter robust linear test for total curcuminoid

- Rho: 3.1409, 3.94, 0.1396
- R^2: 4.772, 4.77, 0.092

Pr < ChiSq approximately predicts the associated probability level after the selected variable has been entered or removed.

Pr: Probability, ChiSq: Chi-square, Rho: association or correlation, R^2: robust Wald-type association test.
Curcuma spp. [29, 40]; however, we did not find any publications concerning DCS and CURSs expressions together in association with curcuminoid yield in turmeric to date. CURS1 and CURS2 expressions were also clustered as a distant group from DCS and CURS3 in this experiment (Fig. 3). Additionally, the gene expression studies alone cannot explain the magnitude and direction of the association between genes (DCS and CURSs) and gene products (curcuminoids). Thus, a more robust association-based test (robust regression) was applied for finding out the magnitude and direction of the association. DCS and CURSs expressions in turmeric rhizome samples at three different growth stages were studied in association with biologically synthesized curcuminoids viz curcumin, demethoxycurcumin, bisdemethoxycurcumin, and total curcuminoid contents in turmeric rhizome samples for identifying any significant association between them. A robust regression analysis using the M-estimator method was used for the association-based test which detects the outliers in the observed dataset and provides more precise information [32]. Association-based tests between the gene expression and curcuminoids revealed that differential expressions of DCS, CURS1, CURS2, and CURS3 are responsible for the differential curcuminoid balance in turmeric rhizome, and their expressions are not always positively associated or correlated with curcuminoid yield. DCS expression had a significant positive but CURS3 expression had a significant negative association with curcumin yield; however, CURS2 expression showed a significant positive association with demethoxycurcumin. The co-regulated expression of CURS1 and CURS2 were also
found significantly associated with demethoxycurcumin content in turmeric rhizome (Table 7). It was also reported that DCS and CURSs were capable of synthesizing curcuminoids through the major curcuminoid biosynthesis pathway in turmeric [19, 20] where CURS3 is capable of synthesizing all three curcuminoids; however, CURS1 and CURS2 are mainly responsible for the curcumin and demethoxycurcumin biosynthesis [20]. The results in this study suggested that the level of expression of DCS and CURSs genes have different individual effects on curcinoid biosynthesis.

The results showed that higher curcuminoid yield in turmeric rhizome is associated with higher DCS and relatively lower CURS3 expressions, and vice versa. Similarly, higher CURS1 expression is associated with lower curcumin yield; however, higher CURS2 expression is associated with a higher demethoxycurcumin in turmeric rhizome, and vice versa (Table 7). This mechanism of co-expression of diketide-CoA synthase and multiple curcumin synthase genes involved in the curcuminoid biosynthesis pathway in turmeric rhizome revealed a significant effect on curcuminoid balance in different turmeric cultivars. Thus, diketide-CoA synthase and multiple curcumin synthase gene expressions are differentially associated with curcuminoid yield in turmeric rhizome, not always positively associated with individual curcuminoid. Further, enzymatic study regarding gene regulation of DCS and multiple curcumin synthases through genetic engineering and biotechnology approach will help to enhance curcuminoid content in turmeric rhizome or in vitro.

**Conclusions**

Gene expressions of diketide-CoA synthase and multiple curcumin synthases involved in the curcuminoid biosynthesis pathway in turmeric rhizome are influenced by genotype and growth stages. DCS and CURS3 expression patterns were closely associated and distinct from CURS1 and CURS2 expression patterns in turmeric rhizome. The association-based tests showed that DCS and CURSs gene expressions are not always positively associated with curcuminoid yield in turmeric rhizome. DCS expression had a significant positive but CURS3 expression had a significant negative association with curcumin, demethoxycurcumin, bisdemethoxycurcumin, and total curcuminoid yield. Curcumin, a major curcuminoid, is also found negatively associated with CURS1 expression. The co-regulated expressions of CURS1 and CURS2 were significantly associated with demethoxycurcumin content in turmeric rhizome. The gene regulation study of DCS co-expressed with multiple curcumin synthases (CURSs) and the overexpression of DCS through genetic engineering approach will help to enhance curcuminoid content in turmeric rhizome or in vitro for medicinal and industrial use.

**Supplementary Information**

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**Table 7** Summary of the association-based tests between gene expression and curcuminoids

| Gene ID | Association test results | Remarks |
|---------|---------------------------|---------|
| DCS     | +, ***                    | DCS has a strong positive association with all the curcuminoids. |
| CURS1   | −, ***                    | CURS1 expression is negatively associated with curcumin. |
| CURS2   | NS                        | CURS2 expression has a positive association with demethoxycurcumin. |
| CURS3   | −, **                     | CURS3 expression has a strong negative association with all the curcuminoids. |
| CURS1 and CURS2 | ¥ ***              | Covariate effect of CURS1 and CURS2 have significant impact on demethoxycurcumin. |

**Remarks**

- NS: non-significant
- +: positive linear association
- −: negative linear association
- ¥: significant at $p < 0.10$
- **: $p < 0.01$
- ***: $p < 0.001$
Abbreviations
CoA: Coenzyme A; CDNA: Complementary deoxyribonucleic acid; 
CURS1: Curcumin synthase 1; CURS2: Curcumin synthase 2; CURS3: Curcumin synthase 3; DCS: Diketide-CoA synthase; HPLC: High-performance liquid chromatography; mRNA: Messenger ribonucleic acid; PCR: Polymerase chain reaction; PKS: Polyketide synthase; RT-qPCR: Reverse transcription quantitative real-time PCR; SAS: Statistical Analysis System; UDG: Uracil DNA glycosylase

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Authors’ contributions
DKA did the laboratory work, data analysis, and manuscript writing. KM guided the laboratory works, interpreted the results, and proofread the manuscript. VP guided the RT-qPCR, curcuminoid analysis, data analysis, and interpretation. RP ensured the funding for the laboratory work and guided the research work. VR and AB assisted during laboratory analysis and manuscript writing. Further, all the authors have read and approved the final manuscript and agreed for submitting the manuscript for publication in the Journal of Genetic Engineering and Biotechnology.

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Availability of data and materials
The dataset supporting the conclusions of this article are included within the article and its additional file: Additional file 1: Table S1, Table S2, and Annex 1.

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Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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