Semi-quantitative expression studies of genes involved in biosynthesis of curcuminoid in *Curcuma caesia* Roxb.

Neha Behar¹, K L Tiwari² and S K Jadhav¹*

¹School of Studies in Biotechnology, Pt. Ravi Shankar Shukla University, Raipur 492 001, India
²Department of Biotechnology, Guru Ghasidas Central University, Bilaspur 495 001, India

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The development of functional genomics, proteomics, metabolomics and bioinformatics tools has given a new facet to plant secondary metabolites study. The biosynthetic pathway involved in the curcuminoids formation was not clear for a long time. The development of turmeric EST database by David Gang's group has formed a landmark for elucidating curcuminoids biosynthetic pathway in *Curcuma longa* and other species of the genus. The present study reports on the expression profiling of genes involved in curcuminoids synthesis in *C. caesia* Roxb. The study involved primer designing from EST/CDS regions of the major genes (CURS, CURS2, CURS3, DCS & CHS1) involved in curcuminoids synthesis, RNA extraction from rhizome (5-month & 10-month-old) and leaves of the plant, cDNA preparation and semi-quantitative expression studies of genes. All the genes showed higher expression in rhizome compared to leaves and, among all the genes, CURS showed maximum expression, followed by DCS, CHS1, CURS2 and least by CURS3. The 5-month-old rhizome showed four-fold higher CURS expression as compared to the 10-month-old. Overexpressing this selective gene in *in vitro* culture by elicitation can help in up scaling the bioactive compound in this plant species.

Keywords: Curcuminoids, expressed sequence tags, functional genomics, genes, rhizome

Introduction

Plants are valuable sources of a variety of chemicals including drugs. The biosynthetic pathway that directs the generation and accumulation of important metabolites in different tissues are not fully understood in many medicinal plants. Thus, one important purpose to investigate medicinal plants is to understand genes and enzymes that govern the biological metabolic process to produce bioactive compounds. Genome wide high throughput technologies based on genomics, transcriptomics, proteomics and metabolomics with bioinformatics and system biology can help reach that goal. The genus *Curcuma* belongs to the family Zingiberaceae and plants accumulate pharmacologically important curcuminoids in their rhizomes. Curcumin and two of its derivatives de-methoxycurcumin and bis-demethoxycurcumin are collectively called as curcuminoids. Despite the importance of turmeric as a major food additive and as a drug in various traditional system of medicine, the molecular and functional analyses of its medicinal value was hampered by lack of tools, such as, expressed sequence tags (EST) and ordered genomic contigs. The biosynthetic pathway involved in the curcuminoid formation was not clear for a long time. But the development of turmeric EST database by David Gang's group, named Aromatic Rhizome Expressed Sequence Tags (ArREST), has opened doors for elucidating curcuminoids biosynthetic pathway in *C. longa* and also proposed that type III polyketide synthases are involved in the pathway¹. These findings can also help to study pathway for curcuminoid biosynthesis studies in other important species of the genus. *C. caesia* Roxb., the lesser known turmeric, is important medicinally and used as folklore medicine for the treatment of inflammation, wounds, cold, cough, fever, pneumonia, bronchitis, asthma, leucoderma, tumors, piles, rheumatic pains, infertility etc. The main bioactive substances which have been reported to exert multiple biological effects are curcuminoid, flavonoids, phenolic and high level of alkaloids, which are widely distributed in plants². The present study involved primer designing of the genes involved in curcuminoid synthesis, RNA extraction from rhizome and leaf of the plant, cDNA preparation and semi-quantitative expression studies of genes involved in different tissue of *C. caesia*.

*Author for correspondence
jadHAV9862@gmail.com
Material and Methods

In Silico Survey of Genes Involved in Curcuminoid Synthesis

All the gene sequences involved in curcuminoid synthesis reported in different plants by different authors were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/). Structural and functional characteristics of genes were compiled and studied. EST (expressed sequence tags) and CDS (coding DNA sequences) based primers were designed by using Batch Primer3, a high-throughput web tool for picking PCR primers (http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi)3.

RNA Isolation and Reverse Transcription

The rhizomes of C. caesia were collected from the medicinal garden of Indira Gandhi Agriculture University, Raipur (Chhattisgarh), India and maintained at the shade house of the School of Studies in Biotechnology, Pt. Ravi Shankar Shukla University, Raipur (Chhattisgarh). The 5-month and 10-month-old plant was used for extraction of total RNA using Trizol reagent (Invitrogen) from leaves and rhizome. The RNA was then quantified using Nanodrop spectrophotometer (ND-1000 UV-Vis spectrometer). About 5000 ng of RNA from rhizome and leaves were reverse transcribed using cDNA Reverse Transcription kit (Fermentas cat # K1612) following the manufacturer's protocol. The cDNA were then stored at –20°C till further use.

Semi-quantitative Gene Expression Studies

In semi-quantitative expression profiling, cDNA were amplified with gene specific primers. The sequence of all the primers are mentioned in Table 1. The PCR was carried out in a 20 µL reaction mixture containing: 2500 ng cDNA, 2.5 µL of 10× Buffer with 1µL of 50 mM MgCl₂, 1 µL of 10 mM of dNTPs, 1 µL of each of 100 ng of forward primer and reverse primer, and 0.5 µL of 5 U of Taq DNA polymerase. The cDNA was denatured at 94°C for 5 min, followed by 35 cycles (94°C for 30 sec, 57-61°C (variable) for 30 sec and 72°C for 1 min), and a final incubation at 72°C for 10 min. The amplified product was analysed through 1% agarose gel. The presence of amplicons and their respective intensity were recorded by densitometer under gel documentation system (Bio-Rad). The relative intensity of amplicons provided basis for quantification of level of expression of gene as high, moderate, low and negligible. The data was normalized using the house-keeping gene, α tubulin.

Results and Discussions

The paradigm of biological research has changed tremendously by recent developments in genomics, high-throughput biology and bioinformatics. Conventional biology was based on empirical, labour-intensive, and time-consuming methods but now new research is driven by automatic and high-throughput methods. Secondary metabolite generation and the genes involved in the pathways can be determined by studying functional genomics in conjunction with bioinformatics4. Moreover these advances also help in unravelling the biosynthetic pathways in lesser known and non-conventional species of the genus, which have not been studied but has traditional importance. This is the first report on expression studies of gene involved in curcuminoid synthesis in C. caesia. The chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs) generates backbones of a variety of plant secondary metabolites including curcuminoids5. In silico analysis of the genes and characterizing them structurally and functionally will help in having better knowledge of genes and metabolite biosynthesis in this plant. All the genes studied belong to chalcone/stilbene synthase family. Behar et al5 in in silico survey of ten genes, namely, CUS6 (Curcuminoi synthase), CURS (Curcumin synthase) and DCS1 (Diketide-CoA synthase), CURS2 and CURS37, CIPKS9 (Chalcone synthase-like protein) and CIPKS10, CHS9 (Chalcone synthase) CHS110 and CHS1210, reported that they could be important for the expression studies, but for primer designing, only five genes were chosen on the basis of its role in biosynthetic pathway and chances of its expression in C. caesia. Therefore, CURS, CURS2, CURS3, DCS and CHS1 genes based primers were designed from their EST/CDS sequences by using Batch Primer3 on-line software for expression studies (Table 1).

<table>
<thead>
<tr>
<th>Gene</th>
<th>EST/CDS sequences ID</th>
<th>Primers (5′-3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CURS</td>
<td>DY384950</td>
<td>ATAACACCCCTCTCTTCTCT AGTGCTCTGTCGTAAAAG</td>
<td>155</td>
</tr>
<tr>
<td>DCS</td>
<td>AB495006</td>
<td>AGGTACCGTGCTCTCTTAC CTTTGAGGTGGAAGGTCAG</td>
<td>232</td>
</tr>
<tr>
<td>CHS1</td>
<td>HM161811</td>
<td>AGGTCAACACGCTCCTTTCT CGTCTCCAAGTGTGGCTTA</td>
<td>269</td>
</tr>
<tr>
<td>CURS2</td>
<td>DY393763</td>
<td>CAAATCTCTAGAGACGAAGGAC AAAGATACCCGCTCCTCACC</td>
<td>129</td>
</tr>
<tr>
<td>CURS3</td>
<td>DY394591</td>
<td>CTTGGAGAAGACGAGGTGA CTTGGTATCTCCTCCACCA</td>
<td>136</td>
</tr>
</tbody>
</table>
Katsuyama et al\textsuperscript{1} revealed the curcumin biosynthetic route in turmeric, in which DCS synthesizes feruloyldiketide-CoA, and then CURS converts the diketide-CoA esters into a curcuminoid scaffold. DCS catalyzes the formation of feruloyldiketide-CoA by condensing feruloyl-CoA and malonyl-CoA. CURS1 and CURS2 showed similar substrate specificity for feruloyl-CoA as a starter substrate, while CURS3 preferred both p-coumaroyl-CoA and feruloyl-CoA as a starter substrate. CHS is superfamily of plant type III PKSs and catalyzes iterative decarboxylative condensations of malonyl unit with a CoA-linked starter molecule to produce structurally diverse pharmaceutically important plant secondary metabolites\textsuperscript{5}. \textit{C. caesia} is a perennial plant, the rhizome remains alive underground throughout the year but the leaves emerge in March–April (onset of summer) and droop and dry out in the month of November–December (onset of winter). Thus mid-point of season (after 5 months in August), in which rhizome and leaves both are in good condition, and harvesting stage (after 10 months in January) was chosen for RNA extraction. These stages were chosen on the basis of observing the growth of the plant throughout the season. The expression of all the specific genes involved in curcuminoids synthesis was normalized with the expression of housekeeping gene (\textit{α}-tubulin). Quantitative estimation of gene expression was done by comparing fluorescent intensity values of the bands as calculated by densitometer in Gel documentation system (Bio-Rad) (Fig. 1).

All the genes showed maximum expression level in rhizome in comparison to in leaves (Fig. 2). Among all the genes, CURS showed maximum expression, followed by DCS, CHS1, CURS2 and the least by CURS3. Further, 5-month-old rhizome showed four times higher CURS expression as compared to 10-month-old rhizome (Figs 3 & 4). The results suggest that the genes are actively involved in curcuminoids formation at the mid time of season when the plant is at the peak of its vegetative stage, while the expression level of CURS decreases in case of fully mature plant at the time of harvesting when fully grown leaves dry out and droop. Thus, CURS gene expresses more at a specific stage and after formation of curcuminoids gets switched off, indicating that these compounds are formed in leaves and then translocated and stored in the storage organ, \textit{i.e.}, rhizome.

It is clear from the above results that the expressions of genes are not only tissue specific but also time specific. A comparative account of differential expression of genes in different tissue type and in two \textit{Curcuma} species, \textit{C. longa} and \textit{C. caesia}, were studied on the basis of available literature. In \textit{C. longa}, all the genes except CURS3 showed high level of expression in rhizome as compared to in leaves, while CURS3 showed equal expression in rhizome as well as leaves. In \textit{C. caesia}, all the genes showed higher expression in rhizome as compared to in leaves. According to biosynthetic pathway proposed by Katsuyama \textit{et al}\textsuperscript{7}, CURS3 is the only gene involved in synthesis of bis-demethoxycurcumin and the lowest expression of this gene in the rhizome and in leaves suggests either this compound is not present in \textit{C. caesia} or in very low amount. Similar observation has also been made in our preliminary phytochemical studies. Katsuyama \textit{et al} also\textsuperscript{7} identified and characterized multiple

\begin{figure}
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\includegraphics[width=\textwidth]{fig1.png}
\caption{Fluorescent intensity values of different genes in rhizome and leaves recorded by densitometer.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Expression of different genes involved in curcuminoid synthesis in rhizome and leaf tissues of \textit{C. caesia}. [\textit{α}-Tubulin in rhizome cDNA (1) & leaf cDNA (2); CURS in rhizome cDNA (3) & leaf cDNA (4); DCS in rhizome cDNA (5) & leaf cDNA (6); CHS1 in rhizome cDNA (7) & leaf cDNA (8); CURS2 in rhizome cDNA (9) & leaf cDNA (10); & CURS3 in rhizome cDNA (11) & leaf cDNA (12)]}
\end{figure}
curcumin synthases from the herb *C. longa* and suggested that the existence of three different type III PKSs in *C. longa* is affected not only by the availability of the substrates, but also by the expression levels of the genes encoding these enzymes. Furthermore, the observed difference in the composition of the curcuminoïd compounds in different cultivars of turmeric can also be accounted for by the above two reasons. With the development of functional genomics, proteomics and metabolomics, many new and powerful tools could be applied to plant secondary metabolism study to improve overall understanding and practical manipulation of plant secondary metabolite production. In conclusion, CURS gene was found to be actively expressed in the rhizome as compared to in leaves and so had been the case with 5-month-old rhizome as compared to 10-month-old rhizome. Thus, CURS is showing spatial and temporal type of gene expression.

**References**


