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RESEARCH ARTICLE

A wound inducible chalcone synthase gene from *Dysoxylum gotadhora* (*DbCHS*) regulates flavonoid biosynthesis

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Abstract

Chalcone synthase (CHS) is a type III polyketide synthase and a key enzyme of the phenylpropanoid pathway that generates precursors for flavonoid biosynthesis. The tree species *D. gotadhora* is known for having an abundance of rohitukine, which has anti-inflammatory and immune-modulating effects. In this study, we used the leaves of *D. gotadhora* to clone *CHS* gene (*DbCHS*). The 1188-bp open reading frame (ORF) was part of the 1373-bp full-length DbCHS clone. Compared to other parts of the plant, *DbCHS* is expressed more in the leaves and fruits. This is linked to anti-microbial action against a panel of microbes in these tissues. The leaves and seeds extracts inhibit *Bacillus subtilis*, *Streptococcus pyogenes*, *Bacillus cereus*, and *Candida albicans*. When a plant is hurt, it leaves its tissues open to attack by microbes. To protect themselves, plants often make chemicals that kill microbes. We found that wounding had a big effect on the production of DbCHS. Based on these tests and the results of phylogenetic analysis and molecular docking, we believe that DbCHS is a wound-inducible enzyme that is needed to make flavonoids, which may give the plant antimicrobial properties.

Keywords Chalcone synthase · *Dysoxylum gotadhora* · *Dysoxylum binectariferum* · Flavonoids · Antimicrobial activity · Secondary metabolites

Abbreviations

CHS	Chalcone synthase
PKS	PolyKetide synthase
RACE	Rapid amplification of cDNA ends
QAE	Quercetin equivalent
ORF	Open reading frame
UTR	UnTranslated regions

Introduction

Flavonoid is a class of secondary metabolites in higher plants that comprise several different sub-groups such as chalcones, flavones, flavonols, and anthocyanins, and have a broad diversity of biological functions (Wen et al. 2020). It includes providing protection against harmful ultraviolet radiation; impart resistance against the attack of plant pathogens and herbivores; play an important role as a signaling molecule in plant–microbe interaction as well as in organogenesis of root nodules; inhibits the transportation of growth hormone, auxin; have a significant role in allelopathic plant–plant interactions and modulation of reactive oxygen species levels; act as an attractant for pollination and also required for the viability of pollens in some plant species (Falcone Ferreyra et al. 2012; Wen et al. 2020). In animal systems, flavonoids are reported to have several therapeutic benefits such as antioxidant, anti-inflammatory, antitumor, anti-proliferative, and pro-apoptotic activities (Waheed Janabi et al. 2020).

Chalcone synthase (CHS), an enzyme of the phenylpropanoid pathway is crucial in the production of flavonoids. CHS is a Type III polyketide synthase (PKS) that enzymatically joins three malonyl-CoA molecules and one p-coumaroyl-CoA molecule to produce chalcones. Chalcones are converted to naringenin, which leads to the manufacture of various flavonoids, including condensed tannins, flavones, flavonols, isoflavones, and anthocyanins (Awasthi et al. 2016c). Many plant species have been examined for

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CHS, including *Oryza sativa*, *Zea mays*, *Grewia asiatica*, *Rheum emodi*, *Coleus forskohlii*, *Medicago sativa*, and others (Awasthi et al. 2016c; Pandith et al. 2019). *Dysoxylum gotadhora* (Buch.-Ham.) Mabb., also known synonymously as *Dysoxylum binectariferum* (Roxb.) Hook.f. ex Bedd., is a member of the Meliaceae family of evergreen trees. The tree is native to Asia, specifically India, Sri Lanka, China, and other regions. It has been found that the trunk bark of *D. gotadhora* contains the highest concentration of rohitukine (ranging from 0.15 to 7.04% by dry weight) (Mohanakumara et al. 2010). Rohitukine is a chromone alkaloid that was first found in the stem bark of the *Amoora rohituka* (Harmon et al. 1979) tree, and then later from the trunk wood of the *D. gotadhora* tree (Mohanakumara et al. 2010; Naik et al. 1988). Later, it was discovered that rohitukine can also be found accumulated in the seeds, leaves, and twigs of the *D. gotadhora* plant. As a result, the seeds and leaves of this plant have the potential to be exploited as an alternative renewable source of rohitukine (Mahajan et al. 2015). It has been shown that rohitukine has both anti-inflammatory and immune-modulatory effects (Naik et al. 1988). In 3T3-L1 and C3H10T1/2 cells, it causes an arrest of cells in the S-phase of mitotic division. This results in an inhibition of adipogenesis in these cells. In rats with hyperlipidemia, rohitukine was shown to have both a lipid-lowering and an antioxidant effect on rats (Lakshmi et al. 2013; Mishra et al. 2018; Varshney et al. 2014). Other chemicals, in addition to rohitukine, have been found to originate from this tree. One of them is 6,7-Di-Ac form, also called dysobinin. 6,7-dihydroxy-1,14-meliacadien-3-one (6,7)-form is another molecule from this plant. There have also been reports of N-oxide derivatives of rohitukine and other chromone alkaloids such as dysoline coming from the *D. gotadhora* plant (Mahajan et al. 2015). A fascinating class of secondary metabolites known as chromone alkaloids, such as rohitukine, are produced by the convergence of several different biochemical pathways (Khadem and Marles 2011). Chromone is an isomer of coumarin. Therefore, there is a good possibility that the phenylpropanoid pathway's intermediates are used in the rohitukine biosynthesis process.

Despite its enormous medical importance, there is no report on the CHS-encoding gene from *D. gotadhora* plant. The expression of *CHS* has been related to the production of significant secondary metabolites that helps plants to withstand various environmental stresses. Furthermore, such metabolites are also responsible for endowing any plant with therapeutic capabilities. Therefore, the current study was performed to isolate and characterize the CHS-encoding gene from *D. gotadhora*.

Material and method

Maintenance and stress treatment of plants

Seeds and different tissues of *D. gotadhora* were collected from Forest Research Institute, Dehradun, Uttarakhand (India). Tissues were stored in RNAlater (Thermo Scientific, USA) until further processing for RNA isolation. Seeds were planted in polybags (18 × 28 cm) containing soil mixture (soil rite–sand–soil) and maintained in green house with average relative humidity (RH%) and temperature (°C) of 71% and 24 °C, respectively. Plant specimens were submitted to Janaki Ammal herbarium, CSIR-IIIM (Acronym RRLH, accession no. 22163) (Mahajan et al. 2015).

Wounding treatment was given to 4 months old plants as described earlier (Rather et al. 2015). Briefly, healthy plants of *D. gotadhora* (4 months old) were selected and aerial portions of the plants were pierced numerous times with a sterile needle to induce wounding stress, with an average of 7–8 stabs per leaf. Untreated plants were used as control. Three plants were taken as replicates for both control and wounding treatment. Leaf tissue was sampled after 3 h, 6 h, 12 h, and 24 h of treatment from each replicate and stored at – 80 °C till further use.

Primer designing

Degenerate primers were used for cloning the core fragment (internal region) of *CHS* gene. Gene specific primers for cloning were designed using Primer3 software. LightCycler Probe Design Software 2.0 (Hoffmann-La Roche, Switzerland) was used for designing primers used for gene expression studies. Primers were tested using PCR for single band amplification. Table 1 summarizes information regarding primers used in the study.

RNA isolation, cDNA synthesis, and rapid amplification of cDNA ends

These protocols have been described earlier (Awasthi et al. 2016a). Briefly, RNA was isolated from frozen plant samples, using TRIzol® (Invitrogen, Life Technologies, USA) and quantified using a spectrophotometer (Thermo Scientific, USA). Post DNase (DNA-free™ kit; Ambion® TURBO DNA-free™, Life Technologies, USA) treatment of RNA, cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, USA) using oligo-dT_{12} primer (FirstChoice® RLM-RACE Kit, Ambion®, Life Technologies, USA). Degenerate primers were used for amplification of the core region (internal fragment) of the gene. This fragment was gel-eluted (Qiaex II gel extraction kit,

Table 1 Nucleotide sequence of primers used in the study

Primer code	Orientation	Primer sequence	T_m	Use in this study
*Db*CHSF	Forward	5′-GCYAARGACTTGGCKGARAWCAACAAGGG-3′	60 °C	Degenerate primers for amplification of core cDNA fragment
*Db*CHSR	Reverse	5′- GCRCTTGACATGTTACCRTAWTCACTYA-3′	60 °C	
*Db*CHS5R	Reverse	5′- CGCGGAAAGTGACGGCTGTGATCTC -3′	65 °C	5′ RACE primer
*Db*CHS3R	Forward	5′- AAGCGAAACTGGGTCTGAAA -3′	58 °C	3′ RACE primer
qRTfwd	Forward	5′-ATTCTCCCTGATTCTGACG-3′	55 °C	qRT-PCR of *DbCHS*
qRTrev	Reverse	5′-TTCCAGTCATTGATGCCG-3′	55 °C	
ActinF	Forward	5′-ATGACATGGAGAAGATCTGGCATCA-3′	55 °C	housekeeping gene control for qRT-PCR
ActinR	Reverse	5′- AGCCTGGATGGCAACATACATAGC-3′	55 °C	

Qiagen, Germany) and sequenced. The sequence was used for designing gene specific primers. First Choice® RLM-RACE kit (Invitrogen, USA) was used for performing Rapid amplification of cDNA ends (RACE) reactions to clone *CHS* from *D. gotadhora* (*DbCHS*), following manufacturer's instructions. 2 µg of DNase treated RNA, isolated from the leaf tissue of *D. gotadhora*, served as template for first strand cDNA synthesis. 3′ RACE-PCR was carried out using oligo(dT) primed cDNA and 3′ RACE outer primer along with 3′ RACE-Gene specific primer (GSP). Similarly, 5′ end of the gene was generated using 5′ RACE outer primer and 5′ RACE GSP along with 5′ RACE inner primer. The 5′ and 3′ RACE amplicons were cloned and sequenced.

Expression analysis

For expression study of *DbCHS*, RNA was isolated from different tissues of *D. gotadhora*. The isolated RNA was DNase treated and cDNA was prepared as described above. The LightCycler® 96 Real Time PCR System (Hoffmann-La Roche, Switzerland) was used for expression analysis. Each PCR reaction (20 µL) contained 1X LightCycler® 480 SYBR Green I Master (Hoffmann-La Roche, Switzerland), 1 µM gene specific expression primers (Integrated DNA Technologies, USA) and cDNA (appropriately diluted). Reactions were done in triplicates. For normalization, *Actin* gene was used as internal control. Relative expression level was measured as described earlier (Awasthi et al. 2016a, c).

Similarly, the expression analysis of *DbCHS* gene was carried out in leaf tissues after wounding at different time intervals: 3 h, 6 h, 12 h, and 24 h, with respect to the control leaves.

Total flavonoid quantification and antimicrobial activity

Total flavonoids were quantified using the aluminum chloride ($AlCl_3$) method with quercetin as standard and expressed as mg quercetin equivalent (mg QAE) per gram dry weight of plant material (Awasthi et al. 2016c). The crude extracts were prepared from 100 mg of dried powder of various tissues of *D. gotadhora* plant. For total flavonoid quantification, the extracts were dissolved in 500 µL of distilled water, followed by the addition of 30 µL of 5% $NaNO_2$ and incubation at room temperature for 5 min. 300 µL of 10% $AlCl_3$ solution was then added and the samples were again incubated at room temperature for about 6 min. Further after adding 200 µL of 1 M NaOH and 200 µL of distilled water to the samples, the absorbance was taken at 510 nm. The experiment was done in three replicates for each sample. Quercetin was used as standard for plotting the calibration curve (10–60 µg; $R^2 = 0.974$).

Well diffusion assay was used for determination of anti-microbial activity. This assay and the microbial strains used for determination of anti-microbial activity have been described earlier (Jamwal et al. 2019). Five bacterial pathogens, including, *Bacillus cereus* (IIIM 25), *Bacillus subtilis* (MTCC 121), *Klebsiella pneumoniae* (ATCC 75388), *Staphylococcus aureus* (MTCC 96), *Streptococcus pyogenes* (MTCC 442), and a pathogenic yeast, *Candida albicans* (ATCC 90028), were used for the assessment of antimicrobial activity of the extracts prepared from various tissues of *D. gotadhora* plant. The pathogenic microbes were inoculated in appropriate broth media [Luria–Bertani (LB) broth for bacteria and potato dextrose (PD) broth for yeast]. 200 µL of actively growing pathogenic cultures (OD_{600nm}-0.6) were spread on Petri plates containing appropriate agar medium. Wells were prepared in the agar medium and filled with 40 µL of tissue extract (10 mg/mL in methanol). The plates were incubated for 24 h and the zone of inhibition was recorded. A bigger zone of inhibition corresponded with higher anti-microbial activity.

Different concentrations of ciprofloxacin and clotrimazole were used as controls.

Sequence analysis, alignment, clustering, modeling and docking

The sequences of the core region (internal fragment), 3′ RACE amplicon and 5′ RACE amplicon were assembled, to generate the full length gene sequence. It was translated in silico to generate the protein sequence. Theoretical molecular weight and isoelectric point of the protein were calculated using the Sequence Manipulation Suite v2 (Stothard 2000). MatGat tool (Campanella et al. 2003) was used for the calculation of similarity and identity with homologous sequences from other plants. Protein alignment was carried out using CLC Genomics software (Qiagen, Germany). Phylogenetic trees were constructed using MEGA software (https://www.megasoftware.net/). Modeling and docking analysis with DbCHS, was carried out as described earlier (Awasthi et al. 2016b) using SWISS-MODEL (Waterhouse et al. 2018) and Maestro, version 9.4, Schrödinger (LLC, New York, 2013), respectively.

Results

Cloning and sequence analysis of DbCHS

Cloning of the *DbCHS* gene, which codes for chalcone synthase, was performed using the tissue taken from the leaves of *D. gotadhora*. In order to acquire a core fragment of approximately 500 base pairs, degenerate primers were utilized. The sequence of the inner-core fragment was used to create 5′ and 3′ RACE primers (Table 1), which were then used in RACE-PCRs to acquire the 5′ and 3′ fragments of the DbCHS cDNA. The resulting amplicon sizes were about 700 bp and 380 bp, respectively (Fig. 1).

The full-length *DbCHS* clone was 1373 base pairs long, and it had an open reading frame (ORF) that was 1188 base pairs. The ORF began with an ATG start codon at position 54 and ended with a TGA stop codon at position 1241

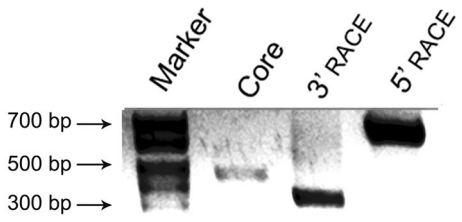

Fig. 1 Cloning of *DbCHS* from *D. gotadhora*: Gel picture shows the PCR amplicons of core fragment, 5′ and 3′ RACE PCR fragments of *DbCHS* with 100 bp DNA marker

(Supplementary Fig. 1). *DbCHS* sequence was submitted to NCBI with GenBank Accession Number: KR703480. *DbCHS* nucleotide sequence coded for a protein of 395 amino acids with molecular weight of 43.05 kDa and pI 6.17. The 5′ and 3′ untranslated regions (UTR) were 53 bp and 132 bp long, respectively. Further, the protein sequence analysis indicated that the conserved motifs and amino acid residues, signature of CHS family, were also found in DbCHS (Supplementary Fig. 1). The 'Cys-His-Asn' catalytic triad (Ferrer et al. 1999; Schröder 1997), characteristic of CHS active site was present at Cys^{164}, His^{303} and Asn^{336} conserved positions in DbCHS (Fig. 2). The other conserved motifs: FGFGPGL, GNMSSA, WIAHPGGPA, LFGDG, and MMYQGCF (Fukuma et al. 2007; Jez et al. 2001; Lanz et al. 1991; Suh et al. 2000) were also present in DbCHS, along with strictly conserved amino acids (Supplementary Figs. 1 and 2).

Secondary structure prediction revealed that DbCHS protein contains α-helix (40%) and random coil (31.39%) predominantly. Extended strand and β-turn had 17.72% and 10.89% prevalence.

The BLASTP program on the NCBI database was used to locate homologs of the DbCHS protein sequence, and then multiple sequence alignment was performed (Supplementary Fig. 2). Sequences of CHS genes were obtained from GenBank: *Dimocarpus longan* Lour. (Sapindaceae) (GenBank Acc. No. 346577498), *Dictamnus albus* L. (Rutaceae) (GenBank Acc. No. 54311699), *Litchi chinensis* Sonn. (Sapindaceae) (GenBank Acc. No. 283827864), and *Theobroma cacao* L. (Malvaceae) (GenBank Acc. No. 590647992). DbCHS sequence shared 93.4% identity and 97.7% similarity with the CHS from *D. longan*; 91.9% identity and 97.7% similarity with *T. cacao*; 92.7% identity and 97.5% similarity with *D. albus*, and 92.4% identity and 97.5% similarity with *L. chinensis* (Fig. 2A).

A phylogenetic tree was also built utilizing CHS protein from various taxa, including bacteria, fungi, monocots, and dicots. CHS is highly conserved gene showing > 80% similarity in higher plants (Mallika et al. 2011). Our analysis also clustered distinctly, the CHS sequences from bacteria, fungi, and plants (Fig. 2B). DbCHS, as expected, belongs to the dicotyledonous plant group, with a tight phylogenetic relationship to CHS from *D. longan*.

Expression analysis of DbCHS, quantification of total flavonoids and antimicrobial activity

Expression analysis of *DbCHS* was performed through real-time PCR in various tissues such as fruit/seed, root, leaf, and stem, to investigate spatial expression patterns. With respect to stem, leaf exhibited maximum expression of *DbCHS*, followed by fruit; on the other hand root had only little expression (Fig. 3A). Further, it was found that

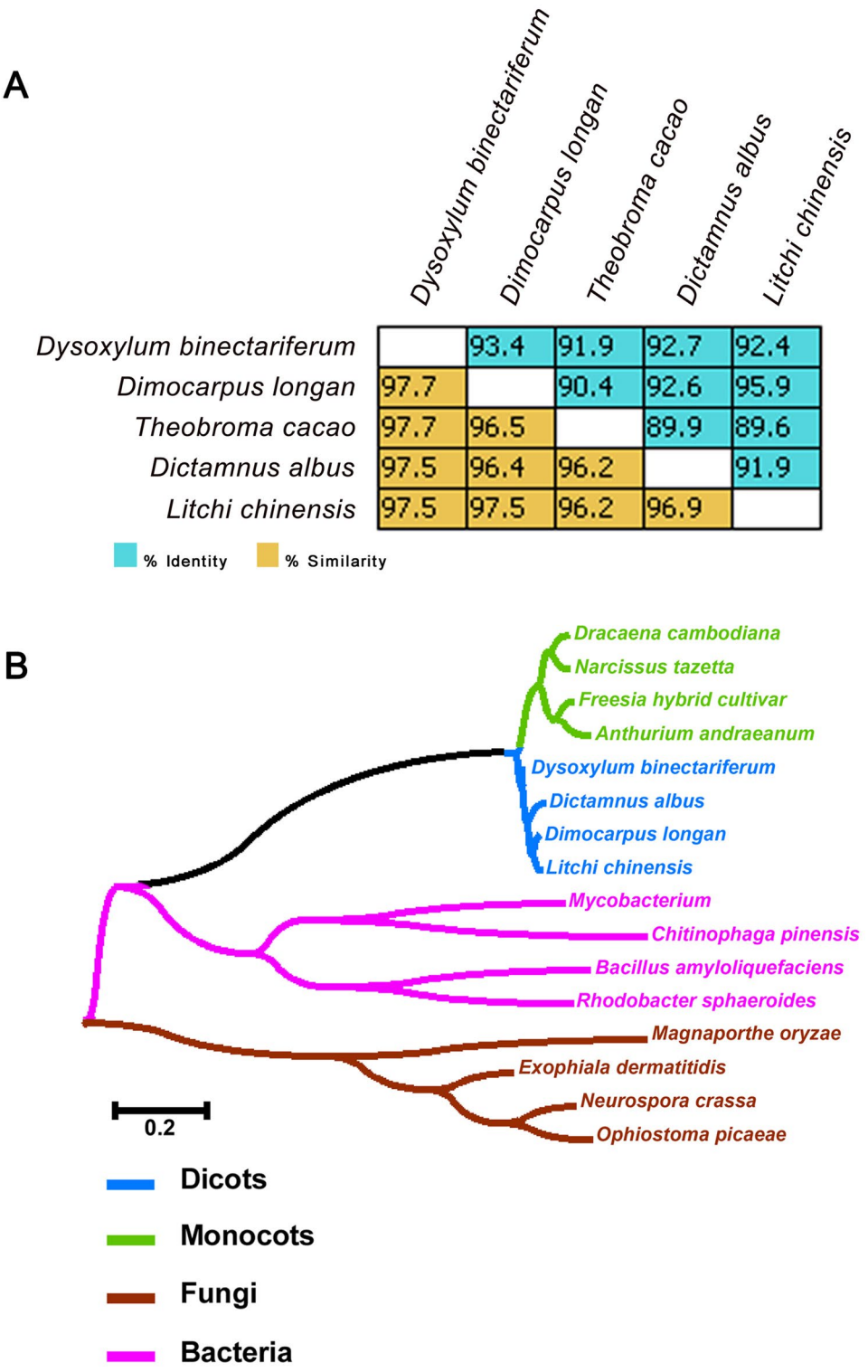

Fig. 2 Similarity of DbCHS with its homologs and phylogenetic analysis: **A** Percentage similarity and identity of DbCHS amino acid sequence and related CHS proteins from different plant species (*D. longon*, *D. albus*, *L. chinensis* and *T. cacao*) **B** Phylogenetic tree of DbCHS with different taxonomic groups including plants, fungi and bacteria, and clustering of DbCHS with closely related CHS homologs from dicotyledonous plants

wounding up-regulated the expression of *DbCHS*, showing up to 8 folds increase at 3 h after treatment, with respect to control (Fig. 3B).

Quantification of total flavonoids in extracts prepared from various tissues of *D. gotadhora* showed the highest content in leaves followed by fruits (Fig. 3C), which corroborates with the expression pattern of *DbCHS*. Total flavonoid content is expressed as mg QAE (quercetin equivalent) per gram dry weight of plant material. A standard curve of quercetin (Fig. 3D) was used for estimating the flavonoid content in extracts (Awasthi et al. 2016a, c).

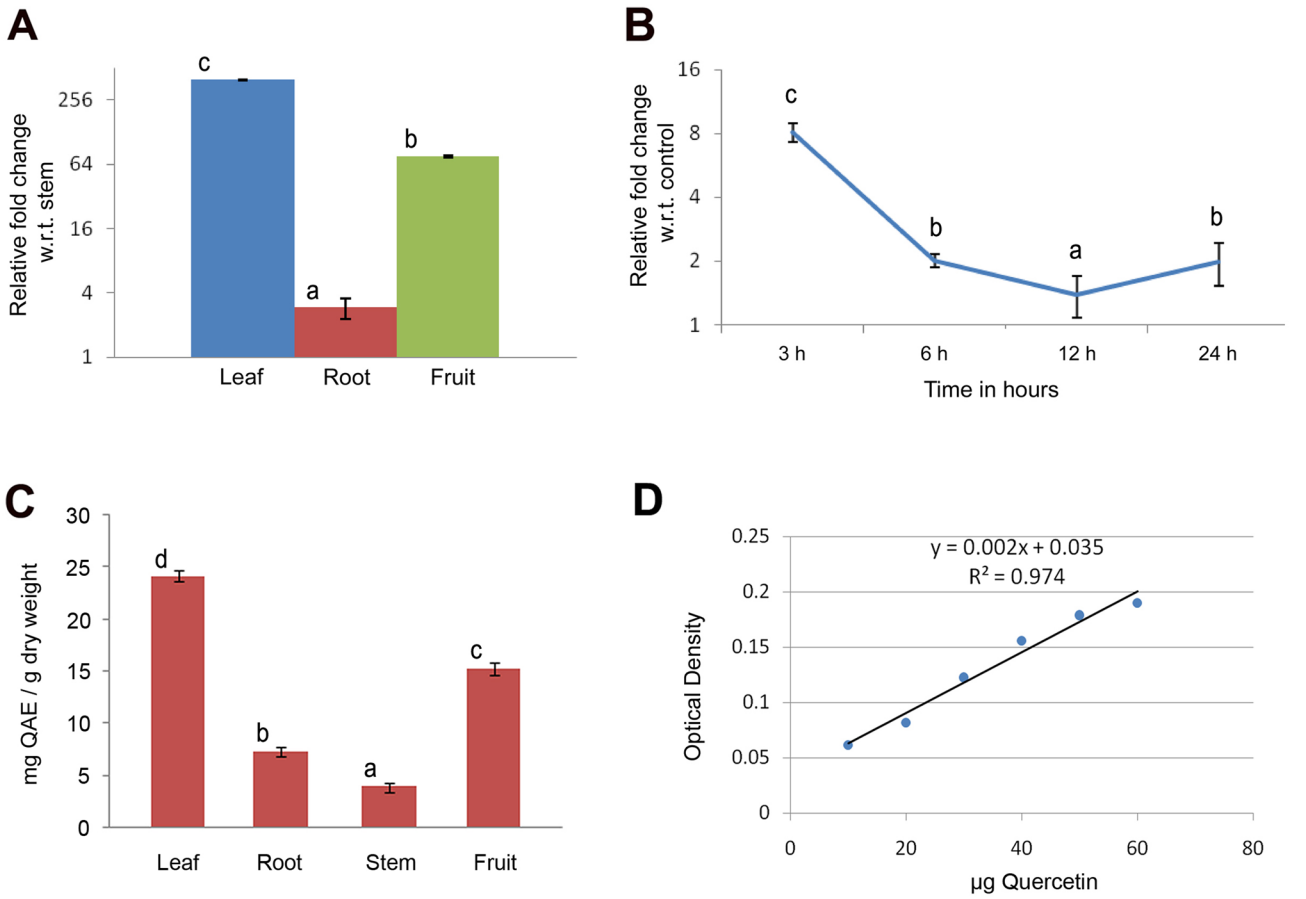

Fig. 3 Expression analysis of *DbCHS* and quantification of total flavonoids **A** Expression of *DbCHS* in different tissues (Leaf, Root and Fruit) of *D. gotadhora* depicted as fold change with respect to expression values in stem **B** Expression of *DbCHS* in response to wounding at different time points (3 h, 6 h, 12 h and 24 h) depicted as fold change with respect to expression values in un-treated control plants **C** Spectrophotometic quantification of total flavonoids in different tissues (Leaf, Root, Stem and Fruit) of *D. gotadhora*, using quercetin as standard **D** Standard curve of quercetin. Expression and quantification data are presented as mean ± standard deviation. Means were compared using one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$). Different superscripts indicate significant differences between the values

Antimicrobial activity of *D. gotadhora* extracts was determined by agar well diffusion assay (Supplementary Fig. 3). It was found that leaf and fruit extracts inhibited the growth of three out of five tested bacterial pathogens, while stem extract inhibited the growth of only one test bacteria (Table 2).

Homology modeling and docking analysis of DbCHS

Closest structurally characterized homolog (PDB ID 4YJY) of DbCHS was found to be a type III PKS from *Oryza sativa* (Poaceae). This was used as a template and a model of DbCHS (Fig. 4A) was built using SWISS-MODEL (Waterhouse 2018). Ramachandran plot analysis of DbCHS, using PROCHECK (Laskowski et al. 1993, 1996), showed that 94% amino acid residues are present in the most favorable region, 5.7% residues in the additional allowed region, 0.3% in the generously allowed region and no residue in the disallowed region, suggesting the acceptability of modeled structure of DbCHS protein (Fig. 4A, B). Physiologically, CHS functions as a homodimeric protein (Pandith et al. 2019; Tropf et al. 1995). However, it was demonstrated experimentally that each subunit is capable of independently carrying out the condensation reactions with the substrates (Tropf et al. 1995). Hence, as described in earlier studies, we used a model of only one chain for docking analysis (Awasthi et al. 2016b; Kumar et al. 2020). Further, in earlier studies also, both substrate and products have been tested using molecular docking analysis (Kumar et al. 2020). CHS utilizes one molecule of coumaric acid and three molecules of malonic acid to produce one molecule of naringenin chalcone which is isomerized to naringenin (Awasthi et al. 2016c; Dao et al. 2011). The binding of likely substrate and product with the model of DbCHS was tested

Table 2 Antimicrobial activities of extracts of *D. gotadhora* (10 mg/mL) against various microbes—*Bacillus subtilis* (MTCC 121), *Bacillus cereus* (IIIM 25), *Klebsiella pneumonia* (ATCC 75388), *Streptococcus pyogenes* (MTCC 442), *Staphylococcus aureus* (MTCC 96), and *Candida albicans* (ATCC 90028) with appropriate antibiotic controls (ciprofloxacin for bacteria and clotrimazole for fungi)

Sample/Test microbe	*Klebsiella pneumonia*	*Bacillus subtilis*	*Streptococcus pyogenes*	*Staphylococcus aureus*	*Bacillus cereus*	*Candida albicans*
	Zone of inhibition (mm)					
Leaf (10 mg/mL)	–	20	17	–	24	14
Root (10 mg/mL)	–	–	–	–	–	12
Stem (10 mg/mL)	–	–	13	–	–	14
Unripe Fruit (10 mg/mL)	–	15	11	–	7	13
Ciprofloxacin (0.25 µg/mL)	15	–	–	18	–	–
Ciprofloxacin (0.5 µg/mL)	15	4	6	20	–	–
Ciprofloxacin (1 µg/mL)	24	8	10	25	–	–
Ciprofloxacin (2 µg/mL)	32	20	20	30	10	–
Clotrimazole (0.25 µg/mL)	–	–	–	–	–	–
Clotrimazole (0.5 µg/mL)	–	–	–	–	–	20
Clotrimazole (1 µg/mL)	–	–	–	–	–	23
Clotrimazole (2 µg/mL)	–	–	–	–	–	27

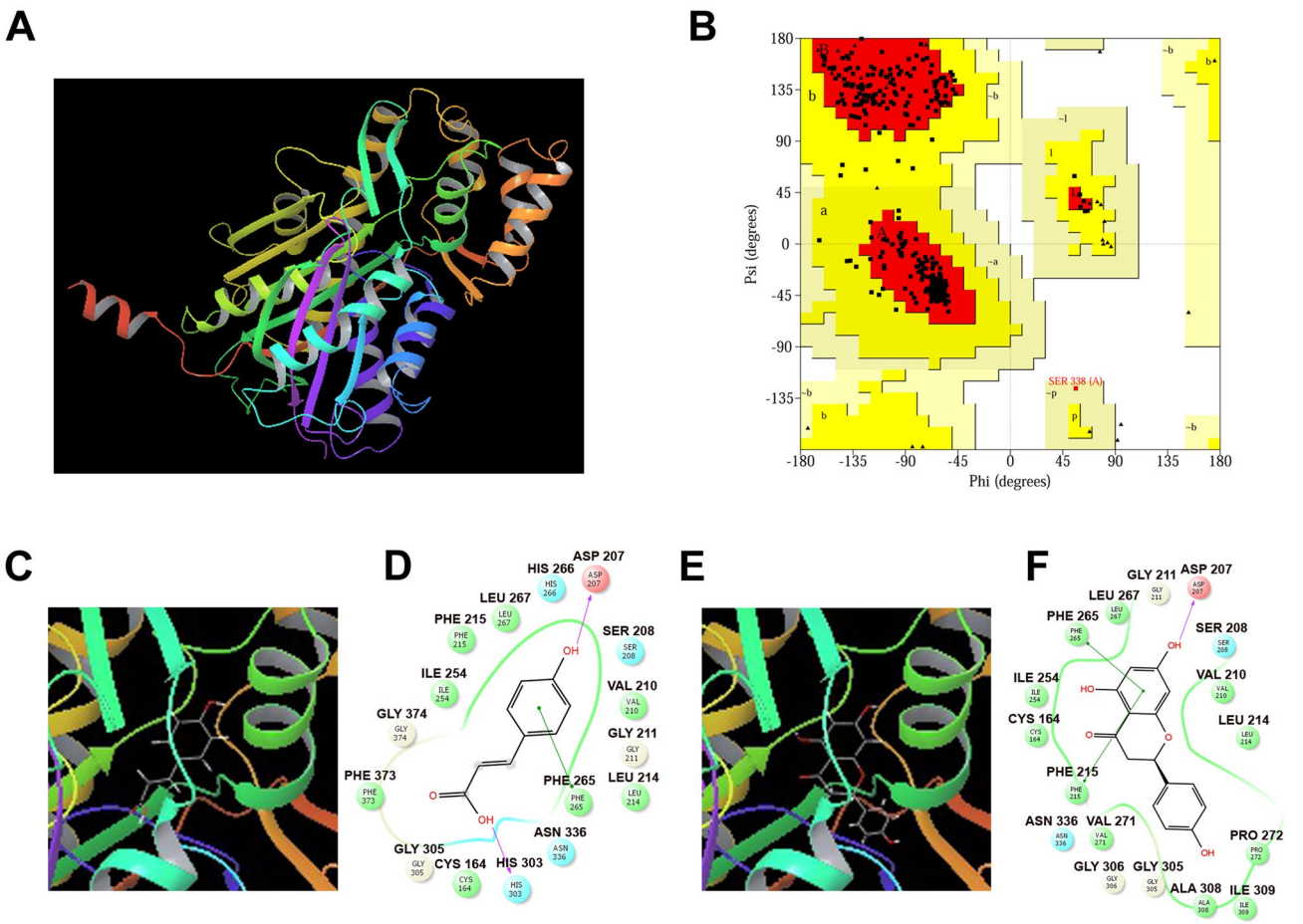

Fig. 4 Molecular modeling and docking analysis of DbCHS **A** Protein model of DbCHS built using SWISS-MODEL **B** Ramachandran plot for the DbCHS protein model using PROCHECK to analyze the protein model **C** Docking of coumaric acid in DbCHS protein **D** Ligand interaction diagram depicting the amino acids involved in the interaction of DbCHS protein with coumaric acid **E** Docking of naringenin in DbCHS protein **F** Ligand interaction diagram depicting the amino acids involved in the interaction of DbCHS protein with naringenin

computationally, using molecular docking analysis. A 10 nm grid was built around the catalytic triad of DbCHS (Cys^{164}, His^{303}, and Asn^{336}) and ligands: coumaric acid and naringenin were docked (Fig. 4C, E). Ligand interaction diagrams (Fig. 4D, F) show the amino acid residues of DbCHS that are involved in interaction with ligands and catalysis.

Discussion

Several studies have reported the cloning of *CHS* gene from different plants. *CHS* cloned from *Saussurea medusa* Maxim. (Compositae) has 1170 bp ORF which codes for 389 aa long polypeptide with molecular weight of 43 kDa (Xia et al. 2011) while CHS from *Aquilaria sinensis* (Lour.) Spreng. (Thymelaeaceae) has ORF of 1192 bp encoding 397 amino acids (Wang et al. 2013). Similarly, CHS containing 1173 bp ORF and encoding 390 aa polypeptide was cloned from *Rhus chinensis* Mill. (Anacardiaceae) (Ma et al. 2015). In 2022, Liu and coworkers reported the cloning of *CHS* gene from *Poncirus trifoliata* (L.) Raf. (Rutaceae) having ORF of 1156 bp encoding 391 amino acids (Liu et al. 2022). Recently, two full-length *CtCHS* genes (*CtCHS1* and *CtCHS3*) were successfully cloned from *Carthamus tinctorius* L. (Asteraceae) having gene lengths of 1525 bp and 1358 bp, respectively (Tang et al. 2023).

Further, the expression results of *DbCHS* gene are comparable to the previous reports, wherein maximum expression of *CHS* was observed in phyllosphere of the plant (Awasthi et al. 2016c; Fritze et al. 1991). Since CHS is involved in the biosynthesis of pigment compounds, a higher expression level of *CHS* was found in flowers and leaves of snapdragon (Fritze et al. 1991). It has been reported that the expression of *CHS* may be upregulated in response to herbivorous attacks, pathogenic infections as well as abiotic stresses such as UV exposure, wounding, or chemical elicitor treatment (Richard et al. 2000; Schenk et al. 2000). In *Coleus forskohlii* (Lamiaceae), methyl jasmonate treatment, that mimics tissue damage (Rather et al. 2015), was found to significantly induce *CHS* expression (Awasthi et al. 2016c). It has been shown earlier that engineered *Ipomoea purpurea* L. (Roth) plants with mutated copies of the *CHS* gene are more prone to *Rhizoctonia solani* infection and losses due to herbivorous attack as compared to the wild type plants, providing evidence that CHS is involved in pathogen resistance (Dao et al. 2011). In the present study, we have analyzed the expression of *DbCHS* in response to wounding and found that the expression of the gene was upregulated at different time points with respect to the control. Several other studies, where the time profile of gene expression in response to different treatments given to plants were investigated, generally compare the expression levels of gene-of-interest at different time points with the expression values at 0 h. However, circadian rhythms are known to affect the expression of numerous genes in plants, including the *CHS* gene (Romanowski et al. 2020; Schaffer et al. 2001). Studies conducted in transgenic *Arabidopsis* plants have reported that *CHS* promoter is regulated by the circadian clock (Thain et al. 2002). Hence, we reasoned that it would be appropriate to compare the *actin*-normalized expression of *DbCHS* at different time points in experimental plants with the normalized expression values of *DbCHS* at the same time points in control plants, for calculating fold change. This would nullify any changes in gene expression that may arise due to the internal circadian rhythm of the plant. However, it is imperative to note that *D. gotadhora* is a sexually reproducing species and slight variation in gene expression could also arise due to genetic variability. In order to circumvent this issue, we have taken three biological replicates in our experimental design.

Studies have reported CHS to be a key enzyme involved in flavonoid biosynthesis. The overexpression of *AeCHS* gene from *Abelmoschus esculentus* in transgenic *Arabidopsis thaliana* plants was reported to increase the production of flavonoids and the expression of other downstream genes of flavonoid biosynthesis pathway (Wang et al. 2018). Further, the transcriptome analysis to understand wounding-induced flavonoids accumulation in *Dalbergia odorifera,* revealed the upregulated expression of the *CHS* gene family along with other gene families of the phenylpropanoid and flavonoid pathways (Sun et al. 2020). In *Senna tora* plant, wounding has been reported to activate the expression of nine CHS-encoding genes along with other flavonoid synthesis genes, which led to the accumulation of flavonols- kaempferol and quercetin. These flavonols are known to possess antimicrobial and antioxidant properties (Kang et al. 2021).

The antimicrobial activities of flavonoids are well established (Adamczak et al. 2019; Cushnie and Lamb 2005; Górniak et al. 2019). Our results also suggest that *DbCHS* is a wound-inducible gene that is involved in the biosynthesis of flavonoids, which imparts antimicrobial activity to *D. gotadhora*. Since wounding predisposes plants to pathogen attack, induction of *DbCHS* after wounding, as observed in our study, may help in protecting plants against pathogen attack by using the products of phenylpropanoid pathway for the production of flavonoids and isoflavonoid phytoalexins. Five flavonoid glycosides isolated from *Graptophyllum granulosum* (Acanthaceae) were shown to be antimicrobial in nature; being active against drug resistant *Vibrio cholera* (Tagousop et al. 2018). Similarly, flavonoid epicatechin has been reported to have a role in protecting avocado fruits against *Colletotrichum gloeosporioides* infections (Guetsky et al. 2005). In sorghum, anthocyanindin flavonoids like luteolinidin and apigeninidin were reported to exhibit antimicrobial activity and were shown to accumulate in response to infection by *Colletotrichum sublineolum* and *C. graminicola* fungi (Poloni and Schirawski 2014). Similarly, in

soybean, the effect of wounding and subsequent infection by *Sclerotinia sclerotiorum* on isoflavonoid content was studied (Wegulo et al. 2005). The concentration of isoflavones such as glycitein, genistein and daidzein increased considerably after five days of infection.

Conclusion

This study reports cloning and analysis of *CHS* gene (*DbCHS*) from *D. gotadhora*. *DbCHS* was found to be expressed more in leaf and fruit tissue of *D. gotadhora* as compared to other tissues. Further, the expression of *DbCHS* was strongly induced in response to wounding. Wounding up-regulated the expression of *DbCHS*, showing up to 8 folds increase at 3 h after treatment, with respect to control. As wounding exposes the plant to microbial attack, induction of *CHS* after wounding, may help in protecting the plant against pathogen attack by enhancing the production of flavonoids. Flavonoids are shown to have antimicrobial properties. In our result, we could detect the highest concentration of flavonoids in leaves and fruits, which matches the expression pattern of *DbCHS*. Further, agar well diffusion assays using extracts prepared from different tissues of *D. gotadhora* showed better antimicrobial activity in leaf and fruit extracts. From our results, we conclude that *DbCHS* is a wound-inducible gene in *D. gotadhora* and the enzyme may help in protecting plants against pathogen attack by utilizing the products of phenylpropanoid pathway for the production of flavonoids and isoflavonoid phytoalexins. Heterologous expression of *DbCHS* in other plants could help in engineering resistance to infections from pathogens.

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Author contributions VM carried out most of the experimental work. NK quantified total flavonoids and determined the antimicrobial activity of extracts. VLJ did the molecular modelling and docking analysis and prepared the figures. RC wrote the manuscript. VLJ assisted in writing. SGG designed the study; supervised VM, NK, VLJ and RC, as well as corrected the manuscript.

Declarations

Conflict of interest Authors declare that they do not have any conflict of interest.

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