RESEARCH ARTICLE

# Cloning and expression analysis of chalcone synthase gene from *Coleus forskohlii*

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## Abstract

Flavonoids are an important class of secondary metabolites that play various roles in plants such as mediating defense, floral pigmentation and plant–microbe interaction. Flavonoids are also known to possess antioxidant and antimicrobial activities. *Coleus forskohlii* (Willd.) Briq. (Lamiaceae) is an important medicinal herb with a diverse metabolic profile, including production of a flavonoid, genkwanin. However, components of the flavonoid pathway have not yet been studied in this plant. Chalcone synthase (CHS) catalyses the first committed step of flavonoid biosynthetic pathway. Full-length cDNA, showing homology with plant *CHS* gene was isolated from leaves of *C. forskohlii* and named *CfCHS* (GenBank accession no. KF643243). Theoretical translation of *CfCHS* nucleotide sequence shows that it encodes a protein of 391 amino acids with a molecular weight of 42.75 kDa and pI 6.57. Expression analysis of *CfCHS* in different tissues and elicitor treatments showed that methyl jasmonate (MeJA) strongly induced its expression. Total flavonoids content and antioxidant activity of *C. forskohlii* also got enhanced in response to MeJA, which correlated with increased *CfCHS* expression. Induction of *CfCHS* by MeJA suggest its involvement in production of flavonoids, providing protection from microbes during herbivory or mechanical wounding. Further, our *in silico* predictions and experimental data suggested that *CfCHS* may be posttranscriptionally regulated by miR34.

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## Introduction

Flavonoids are an important class of secondary metabolites that are involved in a wide array of processes like floral pigmentation, pollination, nitrogen fixation and are produced in response to stress, UV, pathogens, insects etc. (Winkel-Shirley 2002; Cushnie and Lamb 2005; Taylor and Grotewold 2005; Lillo *et al.* 2008; Päsold *et al.* 2010; Fini *et al.* 2011). Several flavonoids have been shown to possess antioxidant, antimicrobial, antiinflammatory or antitumour activities (Cazarolli *et al.* 2008). Chalcone synthase (CHS) is an important enzyme that channels the flux of phenylpropanoid pathway towards biosynthesis of flavonoids (figure 1). Phenylpropanoid pathway provides precursors for biosynthesis of other important metabolites, such as rosmarinic acid, which has been demonstrated to possess antiviral activity against Japanese encephalitis (Swarup *et al.* 2007). CHS belongs to type III polyketide synthase (PKS) superfamily (Schröder 1997). It catalyzes the condensation of three molecules of malonoyl-CoA and one molecule of CoA ester of cinnamic acid or its derivative like coumaric acid yielding naringenin chalcone (Martens and Mithöfer 2005). Chalcone isomerase (CHI) then reversibly converts it to flavanone (Moustafa 1967). These intermediates mark the entry point from where pathway diverges into several branches, each resulting in a different class of flavonoids like

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Praveen Awasthi and Vidushi Mahajan contributed equally to this work.
PA did gene expression profiling using real time PCR, quantification of flavonoid content and measurement of antioxidant activity. VM carried out cloning work and wrote the manuscript. VLJ and NK did the analysis of gene structure and splice variants. YSB and SR provided critical inputs for the study as well as during preparation of manuscript. SGG designed the study, analysed the results and edited the manuscript and figures.

**Keywords.** antioxidant activity; chalcone synthase; elicitors; flavonoid; methyl jasmonate; microRNAs; *Coleus forskohlii*.

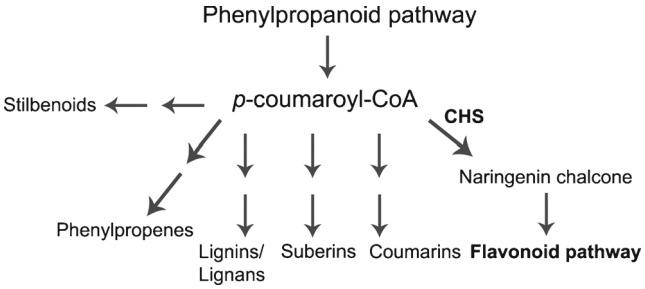


**Figure 1.** Phenylpropanoid pathway. Schematic representation of secondary metabolite biosynthetic pathways depending on *p*-coumaroyl-CoA as precursor. CHS channellizes the flux towards flavonoid pathway.

anthocyanins, phytoalexins and phytoanticipins (Jez *et al.* 2001; Dao *et al.* 2011).

CHS has been studied in many plant species including *Oryza sativa* L. (Poaceae), *Aquilaria sinensis* (Lour.) Spreng. (Thymelaeaceae), *Physcomitrella patens* (Hedw.) Bruch & Schimp. (Funariaceae), *Psilotum nudum* (L.) P. Beauv. (Psilotaceae) etc. (Reddy *et al.* 1996; Goodwin *et al.* 2000; Yamazaki *et al.* 2001; Jiang *et al.* 2006; Wang *et al.* 2013). The number of *CHS* copies varies to a great extent in different plants. A single copy of *CHS* is present in *Antirrhinum majus* L. (Plantaginaceae), six copies in *Ipomoea batatas* (L.) Lam. (Convolvulaceae), eight in *Petunia hybrida* (Solanaceae), two in *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) and *Populus trichocarpa* Torr. & A. Gray (Salicaceae), whereas three copies of *CHS* are present in *Vitis vinifera* L. (Vitaceae) and *Viola cornuta* L. (Violaceae) (Van den Hof *et al.* 2008). *CHS* expression may be induced in response to herbivory, plant pathogens and various abiotic stresses like ultraviolet, wounding or exogenous phytohormone / elicitor treatment (Richard *et al.* 2000; Schenk *et al.* 2000). Dao *et al.* (2011) found that *Ipomoea purpurea* (L.) Roth (Convolvulaceae) plants with nonfunctional copies of CHS suffered almost twice the intensity of infection from *Rhizoctonia solani* and about 25% greater damage from herbivorous attack than the wild type, proving the role of CHS in pathogen resistance. *Coleus forskohlii* (Willd.) Briq. (Lamiaceae) is an important medicinal herb known for the production of forskolin, a potent and reversible activator of adenylate cyclase, responsible for antihypertensive property of the herb (Alasbahi and Melzig 2010). The plant is distributed widely in various regions of Asia such as Sri Lanka, India, Nepal, Bhutan, Thailand and China as well as in Africa and Brazil (Kavitha *et al.* 2010). The plant has a diverse metabolic profile (Kavitha *et al.* 2010; Paul *et al.* 2013), including production of minor compounds such as the flavonoid: genkwanin (7-O-methylapigenin) (Alasbahi and Melzig 2010). However, components of the flavonoid pathway have not yet been studied in this plant. Here, we carried out the isolation of the gene encoding CHS, *CfCHS*, from leaf of *C. forskohlii*. Quantitative real time RT-PCR was used to investigate its expression in different tissues and under the effect of various abiotic elicitors.

## Materials and methods

### *Plant material*

*Coleus forskohlii* plants were collected on 17 October 2011 and identified by Dr Y. S. Bedi. A voucher specimen was prepared and submitted to Janaki Ammal herbarium, IIIM (Acronym RRLH, accession no. 22164). Plants were grown and maintained in greenhouse.

### *RNA isolation and cDNA synthesis*

Total RNA was isolated from the young leaves, mature leaves, stem, root and root tip of plants using TRIzol® (Invitrogen, Life Technologies, USA), following manufacturer's instructions. Quantity and quality of RNA was assessed using NanoDrop spectrophotometer (Thermo Scientific, USA) and agarose gel electrophoresis (2%), respectively. Total RNA was treated with DNase (DNA-free™ kit; Ambion® TURBO DNA-free™, Life Technologies, USA) to remove any potential DNA contamination. cDNA was synthesized by using ImProm-II™ Reverse Transcription System (Promega, Madison, USA) with an anchored oligo-$dT_{12}$ primer (FirstChoice® RLM-RACE Kit, Ambion® , Life Technologies, Carlsbad, USA) and 1 $\mu$g of DNAse treated RNA as template.

### *Cloning of full-length CfCHS*

Degenerate primers (CfCHSF and CfCHSR; refer table 1 for sequences) were used to obtain partial clone of *CfCHS* by carrying out PCR amplification from leaf cDNA in a total volume of 20 $\mu$L reaction mixture containing 2 $\mu$L buffer (10×; with $MgCl_2$), 2 $\mu$L dNTPs (2 mM), 1 $\mu$L each primer (5 $\mu$M), 1 unit of *Taq* DNA polymerase (New England Biolabs, USA) and cDNA template under the following conditions: 10 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 60°C and 40 s at 72°C, followed by a final extension of 72°C for 10 min. A 569-bp DNA fragment was obtained and gel elution was carried out following manufacturer's protocol (Qiaquick gel extraction kit; Qiagen, The Netherlands). The eluted product was cloned into the pTZ57R/T plasmid (InsTAclone™ PCR Cloning Kit; Fermentas, Thermo Fisher Scientific, USA) and sequenced. The sequence thus obtained was used to design gene-specific primers (GSP) for carrying out 3′ and 5′ RACE.

GSP CfCHS3R and 3′ RACE outer primer (provided in the kit) were used for 3′ RACE-PCR reaction. Using primary PCR product as template, nested PCR was performed with GSP CfCHS3R forward primer and 3′ RACE inner primer. Similar procedure was followed for obtaining 5′ end sequence using First Choice® RLM-RACE kit (Ambion®, Life Technologies, USA), where GSP CfCHS5R with 5′ RACE outer primer and GSP CfCHS5R with 5′ RACE inner primer were used for the primary and nested PCR reactions, respectively (table 1). Thermal profile for nested PCR was as follows: 10 min at 95°C, 35 cycles of 30 s at 95°C, 30 s

**Table 1.** Nucleotide sequence of primers used in the study.

| Primer | Primer code | Primer sequence | $T_m$ (°C) |
|---|---|---|---|
| Degenerate primer forward | CfCHSF | 5′-CGGAATTCATGATGTAYCARCARGGBTGCTTYGC-3′ | 60 |
| Degenerate primer reverse | CfCHSR | 5′-GCTCTAGACRCAIGCRCTBGACATRTTICC-3′ | 60 |
| 5′ RACE primer | CfCHS5R | 5′-GGAAGGTGACGGCGGTGATCTCGG-3′ | 65 |
| 3′ RACE primer | CfCHS3R | 5′-GCTGAGTGAGTACGGCAACATGTC-3′ | 60 |
| Full-length forward | CDSfwd | 5′-ATGGTGACCGTGGAGGGGATCCGCC-3′ | 60 |
| Full-length reverse | CDSrev | 5′-TCAGTTGATGGGCACACTGTGAAGC-3′ | 60 |
| qRT forward primer | QRTfwd | 5′-CTGGACGCGCGGCAGGATAT-3′ | 65 |
| qRT microRNA forward primer | RTMiRNA 34 | 5′-GGGTTGGTCGATTGGTGTGACGGT-3′ | 63.4 |
| qRT microRNA forward primer | RTMiRNA 166 | 5′-GCGGACCAGGCTCTAGTGGTGT-3′ | 63.3 |
| qRT microRNA forward primer | RTMiRNA395 | 5′-GGCGCACTTCACGAACTTCCCTTG-3′ | 62.5 |
| qRT microRNA forward primer | RTMiRNA477 | 5′-CCCGGGAGGAGTTTCTGAAGGAGAA-3′ | 61.8 |
| qRT microRNA internal control forward primer | RT5.8S F | 5′-CAACGGATATCTCGGCTCTC-3′ | 58.2 |
| qRT microRNA internal control forward primer | RT5.8S R | 5′-TTGTGACACCCAGGCAGAC-3′ | 58.61 |
| qRT internal control forward primer | RT actin F | 5′-CCGTGGAGAAGAGCTACGAG-3′ | 56.9 |
| qRT internal control reverse primer | RT actin R | 5′-TCACACTTCATGATGGAGTTGTAGG-3′ | 56.5 |

at $T_m$ and 1 min at 72°C followed by final extension at 72°C for 10 min. Amplified DNA fragments obtained from 3′ and 5′ RACE reactions were cloned in pTZ57R/T plasmid (InsTA Clone PCR cloning kit; Fermentas) and sequenced. Full-length coding DNA sequence (CDS) of *CfCHS* was amplified from leaf cDNA using primers (CDSfwd and CDSrev, table 1) designed from the sequences of 3′ RACE and 5′ RACE amplicons. These primers were also used with genomic DNA as template for testing the presence of an intron. All primers were obtained from Integrated DNA Technologies, USA.

### *Sequence analysis*

The partial nucleotide sequences obtained for the core fragment, 3′ and 5′ RACE products were assembled using the assembly tool in CLC Genomics Workbench (Qiagen, The Netherlands). Open reading frame (ORF) in the assembled full-length *CfCHS* sequence was predicted by using ORF finder tool (www.ncbi.nlm.nih.gov/gorf/gorf.html). The nucleotide sequence of *CfCHS* was translated using ExPASy translate tool (Gasteiger *et al.* 2003). Computational analysis was carried out to predict potential miRNA-binding sites using psRNATarget tool (Dai and Zhao 2011). For miRNA target prediction, complete nucleotide sequences (with UTRs) of *CfCHS* and its homologues from *Solenostemon scutellarioides* (L.) Codd (Lamiaceae), *Agastache rugosa* (Fisch. & C. A. Mey) Kuntze (Lamiaceae), *Perilla frutescens* (L.) Britton (Lamiaceae) and *Misopates orontium* (L.) Raf. (Plantaginaceae) were analysed (maximum expectation was set to 5.0, all other parameters were set as default). Multiple sequence alignment with protein sequences from related plants was performed using the CLC genomic workbench (CLC bio, a Qiagen company, The Netherlands). Percentage similarity and identity among the homologous sequences was calculated using MatGAT tool (Campanella *et al.* 2003). Sequences of CHS homologues were downloaded from NCBI GenBank: *A. rugosa* (GenBank acc. no. AFL72079.1), *S. scutellarioides* (GenBank acc. no. ABP57071.1), *P. frutescens* (GenBank acc. no. BAA19548.1) and *M. orontium* (GenBank acc. no. CAJ44127.1). Phylogenetic tree was constructed using MEGA 5.2 using neighbour-joining algorithm (Saitou and Nei 1987; Tamura *et al.* 2004, 2011). The theoretical isoelectric point (pI) and molecular weight of the protein were predicted using ComputePI tool on ExPASy web server (Gasteiger *et al.* 2003).

### *Stress treatment*

Gene expression analysis of *CfCHS* was carried out in plants treated with various elicitors as described earlier for expression study of cytochrome P450 genes in *C. forskohlii* (Awasthi *et al.* 2015). A 50 mL solution of salicylic acid (SA) (1 mM), MeJA (100 $\mu$M), abscisic acid (ABA) (50 $\mu$M) or 2,4-dichlorophenoxyacetic acid (2,4-D) (50 $\mu$M) was sprayed on aerial parts of the potted four-weeks old *C. forskohlii* plants. MeJA-treated plants were covered with a thin, transparent plastic bag and kept in isolation. Plants sprayed with 50 mL of 0.1% ethanol were used as control. Young leaves were sampled for 4 h after treatment. The samples were frozen in liquid nitrogen, and stored at −70°C until further use.

Estimation of flavonoid content and radical scavenging activity were carried out in control and MeJA-treated plants. For this, the plants were treated with MeJA as per the procedure followed for the treatment of plants for gene expression analysis, however, here the treatment was given once a day for three consecutive days, following which the plant samples were harvested and shade-dried. Crude extracts were prepared from 100 mg of dried leaves, stems and roots of *C. forskohlii*. Tissues of shade dried were powdered and

extracted thrice with methanol at 30°C (with sonication) for 3 h. The crude extracts thus obtained were analysed using spectrophotometry.

#### *Expression analysis using qRT-PCR*

Quantitative real time RT-PCR (qRT-PCR) was carried out for expression profiling of *CfCHS* in different tissues and under different elicitor treatments as described earlier (Rather *et al.* 2015). Briefly, first strand cDNA synthesis was performed using ImProm-II™ Reverse Transcription System (Promega, USA). Primers used for qRT-PCR study were designed using Primer3 software (Untergasser *et al.* 2012). qRT-PCR assay was carried out using the LightCycler® 96 Real Time PCR System (Hoffmann-La Roche, Switzerland) according to the manufacturer's instructions. Each PCR reaction (20 $\mu$L) contained 1× LightCycler® 480 SYBR Green I Master (Hoffmann-La Roche, Switzerland), 1 $\mu$M primers (Integrated DNA Technologies, USA, refer to table 1 for sequences: qRTfwd and CfCHS5R) and cDNA (appropriately diluted). Thermal cycling conditions for the qPCR were: preincubation at 95°C for 10 min, followed by 45 cycles of 3 step amplification (95°C for 10 s, 60°C for 15 s and 72°C for 25 s). The PCR was followed by a dissociation curve analysis (heating to 95°C for 10 s at normal ramping, cooling to 65°C for 60 s at normal ramping followed by slow heating to 97°C for 1 s at reduced ramping rate of 0.2°C/s) to test qPCR reaction specificity. Each assay was carried out in triplicate and a nontemplate negative control was included. *Actin* gene was chosen as house-keeping internal control for normalization. The threshold cycle ($C_t$) of the amplification curve was used for the calculations. The relative expression level was analysed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001; Awasthi *et al.* 2015), where $\Delta\Delta C_t = (C_{t,\ target} - C_{t,\ actin})_{time\ x} - (C_{t,\ target} - C_{t,\ actin})_{time\ 0}$.

#### *MicroRNA expression study using RT-PCR*

Total RNA of 1 $\mu$g was tailed and reverse transcribed by NCode™ Express Sybr® GreenER™ miRNA qRT-PCR kit, universal (Invitrogen, Life Technologies, USA) according to the user's manual. MiRNA specific primers (table 1) were used to estimate their expression in response to MeJA and ABA. $C_t$ values were normalized to 5.8S gene. Relative miRNA expression was calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001).

#### *Quantification of total flavonoid content*

Total flavonoid content was quantified using spectrophotometer as described earlier (Kaur *et al.* 2013). Briefly, dried crude extract (prepared from 100 mg of dried plant material) dissolved in 500 $\mu$L of distilled water was mixed with 30 $\mu$L of 5% $NaNO_2$ solution, followed by 5 min of incubation at room temperature. After this, 300 $\mu$L of 10% $AlCl_3{\cdot}H_2O$ solution was added and the sample was further incubated for 6 min. Finally, 200 $\mu$L of 1 M NaOH and 200 $\mu$L of distilled water were added to the sample and absorbance was read at 510 nm. Total flavonoids were calculated using quercetin as standard (10–100 $\mu$g; $R^2 = 0.998$). The results were expressed as mg quercitin equivalent (mg QAE) per gram dry weight of plant material. The experiment was done in triplicates.

#### *DPPH radical scavenging activity*

Measurement of radical scavenging activity of *C. forskohlii* in response to MeJA treatment was carried out in different tissues (leaves, stem and roots) according to the method described by Blois (1958). Ascorbic acid was used as positive control and per cent inhibition was determined according to the following equation:

$$\%\text{Inhibition} = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100,$$

where $A_S$ is the absorption of the solution when the sample extract was added at a particular concentration and $A_{DPPH}$ is the absorbance of the DPPH solution. Three experimental replicates were taken for the assay. $IC_{50}$ values were calculated as the concentration of extracts causing 50% inhibition of DPPH radical, a lower $IC_{50}$ value corresponded to a higher antioxidant activity of sample.

## Results and discussion

#### *Cloning of CfCHS and sequence analysis*

Degenerate primers were used to obtain a core fragment of 569 bp. Using the sequence information of core amplicon, 5′ and 3′ RACE primers were designed. RACE-PCR was carried out to obtain the 5′ and 3′ ends of the cDNA, giving an amplicon size of 842 and 362 bp, respectively (figure 2). The full-length clone of 1598 bp contained an ORF of 1176 bp, starting with an ATG start codon at position 245 and ending with a TGA stop codon at position 1420. The clone was sequenced and designated as *CfCHS* (NCBI GenBank accession no. KF643243). CDS primers yielded an amplicon of about 1500 bp from the genomic DNA template, suggesting the presence of intron(s) in *CfCHS* gene

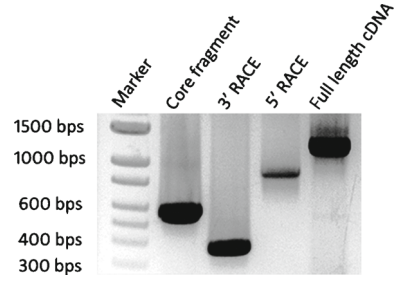


**Figure 2.** Cloning of *CfCHS*. Cloning of *CfCHS* gene from *C. forskohlii*. Gel picture shows the PCR amplicons of core fragment, 5′ and 3′ RACE PCR fragments and full-length *CfCHS* coding DNA sequence.

(figure 3). Further, an amplification of full-length CDS was attempted from cDNAs of different tissues. In conventional end-point PCR, bands were detected only in young leaf and root tissues and they were of the same size, suggesting absence of alternative splicing (figure 4). Moreover, in general, CHS genes from different plants are known to contain only a single intron flanked by two exons (Van den Hof *et al.* 2008). Theoretical translation of *CfCHS* nucleotide sequence encoded a protein of 391 amino acids having a molecular weight 42.75 kDa and pI 6.57 (figure 5). The 5′ and 3′ untranslated regions (UTR) were 244 and 178-bp long, respectively. Closest homologues of CfCHS protein sequence were identified using BlastP (NCBI) and multiple sequence alignment was carried out (figure 6). CfCHS sequence shared 92.6% identity and 94.9% similarity with the CHS from *A. rugosa* (figure 6b). A phylogenetic tree was constructed using CHS protein sequences from different organisms, and as expected, CfCHS fell in the clade of dicotyledonous plants showing close phylogenetic similarity with CHS from *S. scutellarioides*, belonging to the same family (figure 7).

### *Expression analysis*

To understand the spatial pattern of distribution, expression profiling of *CfCHS* was carried out in flower, young leaf, mature leaf, stem, root and root tip using qRT-PCR. Young leaf showed highest expression of *CfCHS*, followed by flower, while root and root tip showed negligible expression (figure 8a). The data is consistent with the previous studies, where highest expression is observed in aerial parts of the plant (Fritze *et al.* 1991; Thain *et al.* 2002). CHS plays an important role in flower pigmentation (Napoli

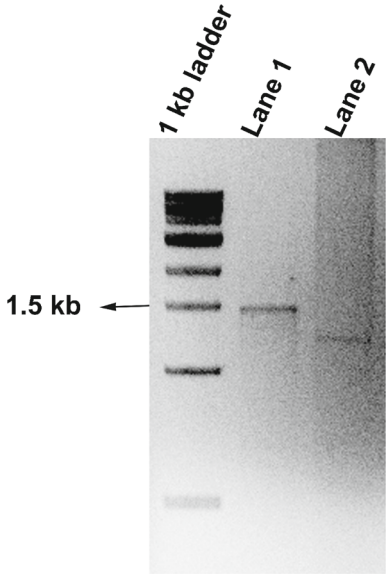


**Figure 3.** Amplification of *CfCHS* from genomic DNA and cDNA of *C. forskohlii*. Lane 1, amplified *CfCHS* gene from genomic DNA using CDSfwd and CDSrev primers. Lane 2, amplified *CfCHS* gene from cDNA using CDSfwd and CDSrev primers.

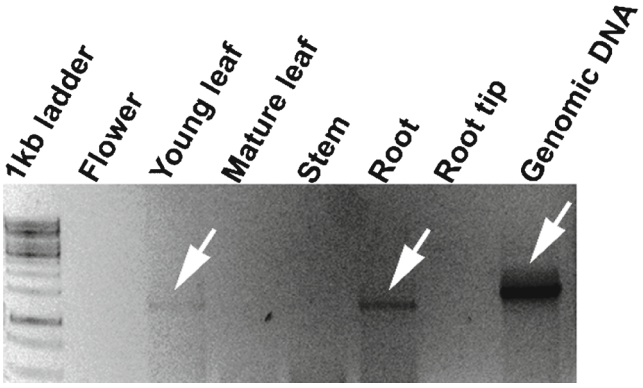


**Figure 4.** PCR amplification of full-length CDS of *CfCHS* gene using cDNA template from different tissues of *C. forskohlii*.

*et al.* 1990; Vander Krol *et al.* 1990). Maximum accumulation of CHS was demonstrated in the corolla and young leaves of snapdragon, where pigmentation is intense (Fritze *et al.* 1991).

Treatments with chemical elicitors are known to mimic the effects of environmental stresses (Tuteja and Sopory 2008). The changes in expression pattern of *CfCHS* on treatment with various phytohormones/elicitors like MeJA, SA, ABA and 2,4-D were also determined. *CfCHS* expression was strongly induced in plants treated with MeJA (figure 8b), which mimics herbivore attack or mechanical wounding (Creelman and Mullet 1997; Kessler and Baldwin 2002). MeJA can diffuse to distal parts of plant (Karban *et al.* 2000; Ruiz-Medrano *et al.* 2001), thereby mediating systemic signalling. Wounding exposes the plant to pathogenic bacteria and fungi. Flavonoids are reported to have antimicrobial properties (Cushnie and Lamb 2005; Orhan *et al.* 2010). Thus, the enhanced expression of *CfCHS* involved in flavonoid biosynthesis in response to MeJA would probably result in accumulation of flavonoids that may protect the plant from pathogenic microbes. It has been reported that MeJA promoted silymarin production and enhanced CHS activity in cell cultures of *Silybum marianum* (L.) Gaertn. (Asteraceae) (Sánchez-Sampedro *et al.* 2005). MeJA also induced *CHS* expression in *Petunia* corollas (Tamari *et al.* 1995). Abscisic acid is another important phytohormone that serves myriad functions ranging from developmental to adaptive stress responses (Cutler *et al.* 2010). ABA promoted synthesis of flavonoids and *CHS* expression in *Ginkgo biloba* (L.) (Ginkgoaceae) (Yan 2002; Shuiyuan *et al.* 2004; Li *et al.* 2014). In our studies, we found that ABA treatment marginally induced expression of *CfCHS* at the concentration of ABA and time-point we tested (figure 8b). 2,4-D is a synthetic auxin herbicide which suppresses anthocyanin (class of flavonoids) synthesis (Takeda *et al.* 1993; Ban *et al.* 2003). However, it has also been reported that anthocyanin synthesis once induced by light could not be suppressed by further addition of 2,4-D (Takeda 1990). In our results also, we observed that 2,4-D had a negligible effect on expression of *CfCHS* (figure 8b). However, *CfCHS*

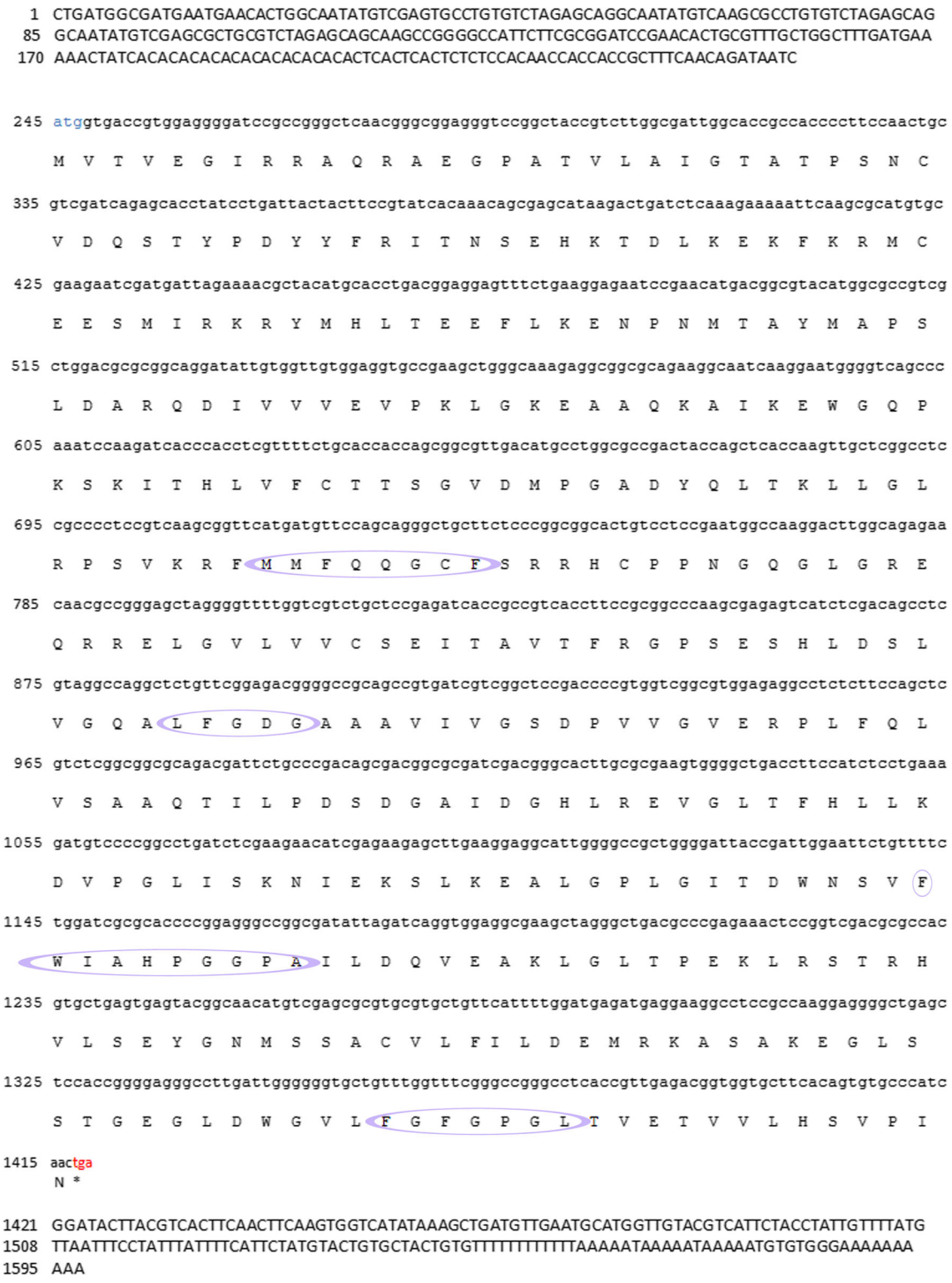


**Figure 5.** Sequence of *CfCHS* from *C. forskohlii*. Nucleotide and the deduced amino acid sequence of CfCHS from *C. forskohlii*. The ATG start codon at position 245, the TAA stop codon at position 1420 and conserved regions are highlighted. Letters in bold face indicate strictly conserved amino acids.

appeared to be downregulated in response to SA treatment (figure 8b). Our results are in agreement with the previous studies performed in constitutive salicylic acid producing transgenic *Nicotiana tabacum* cv. Samsun NN (Solanaceae) plants, where *CHS* was suppressed, relative to the wild-type plants (Nugroho *et al.* 2002).

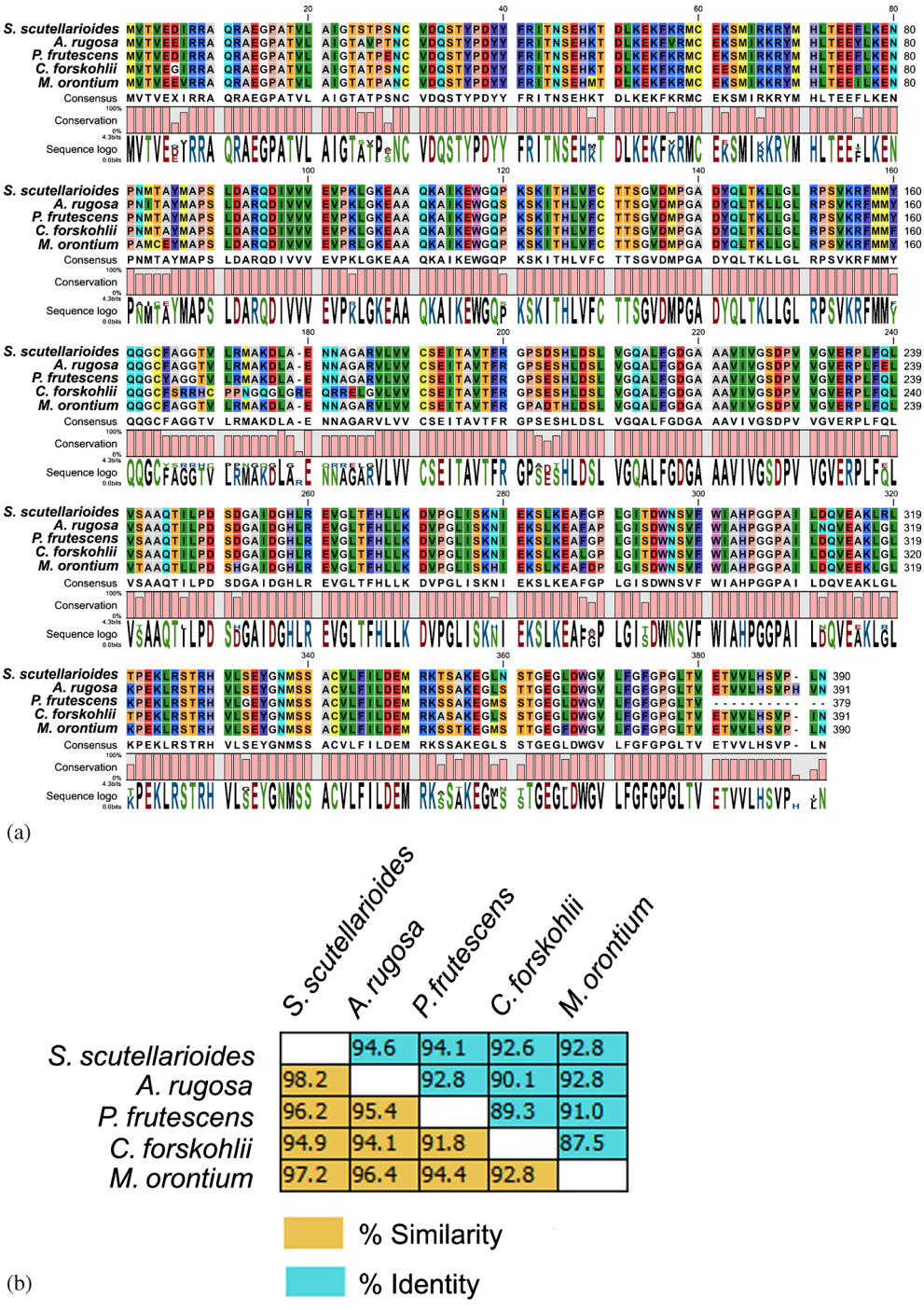


**Figure 6.** Multiple sequence alignment of CfCHS sequence and its homologues in other plant species. (a) Multiple sequence alignment of CfCHS protein sequence with the homologous proteins from other species *A. rugosa* (GenBank acc. no. AFL72079.1), *S. scutellarioides* (GenBank acc. no. ABP57071.1), *P. frutescens* (GenBank acc. no. BAA19548.1) and *M. orontium* (GenBank acc. no. CAJ44127.1). (b) Represents percentage similarity and identity among the related CHS proteins of different plant species calculated using MatGAT tool.

***Total flavonoid content***

Flavonoids significantly contribute to the antioxidant property of the plants (Luo *et al.* 2002). Total flavonoid content in leaves and roots was found to be significantly increased in response to MeJA treatment, whereas there was negligible change in stem (table 2). Significant increase in *CfCHS* gene expression as well as the total flavonoid content in leaves of *C. forskohlii* in response to MeJA treatment suggests that *CfCHS* could be involved in biosynthesis of flavonoids. MeJA has been earlier reported to enhance flavonoid

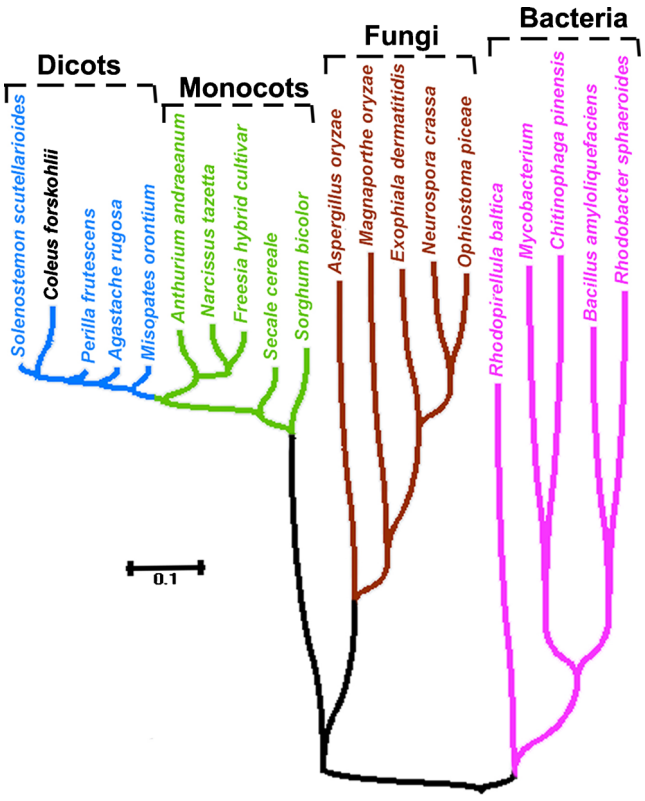


**Figure 7.** Phylogenetic tree of CHS and its homologues. Phylogenetic tree of CfCHS, showing clustering of sequences from different taxonomic groups of plants, fungi and bacteria. CfCHS clusters with related sequences from dicotyledonous plants.

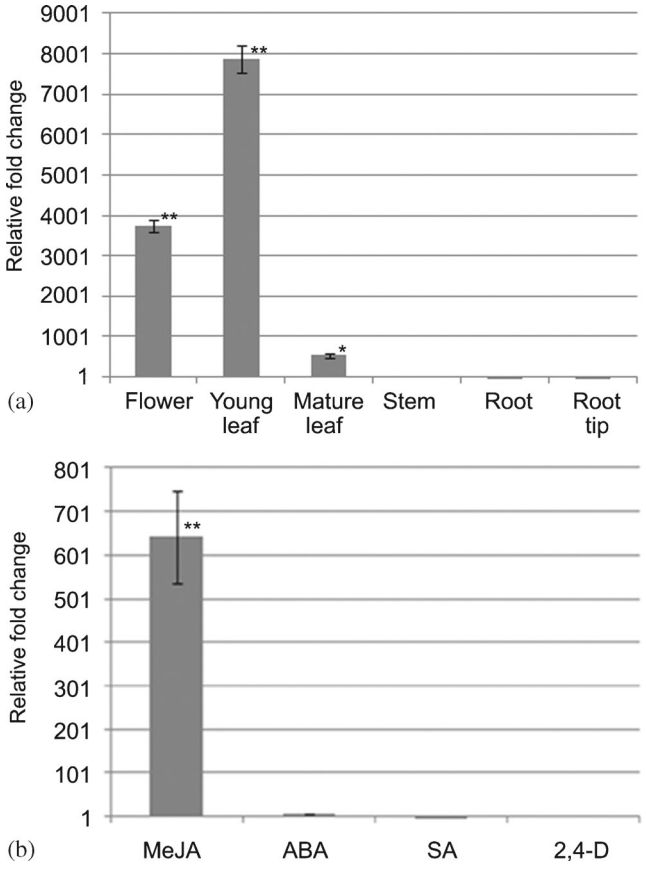


**Figure 8.** Expression profile of *CfCHS*. Expression profile of *CfCHS* (a) in different tissues and (b) under the effect of elicitors. Methyl jasmonate (MeJA), salicylic acid (SA), abscisic acid (ABA) and 2,4-diphenylphenoxyacetic acid (2,4-D) at 4 h after treatment. Actin was used as housekeeping control. Expression of *CfCHS* in stem was used as baseline for calculating fold change in (a). Expression of *CfCHS* in 0.1% ethanol-treated (control) plants was used as baseline for calculating fold change in (b). Three replicates were used for analysis. $*P<0.05$ and $**P<0.01$.

content and antioxidant activity of *Rubus fruticosus* L. (Wang *et al.* 2007).

### *DPPH radical scavenging activity*

DPPH method is a simple, rapid, sensitive and reproducible assay used for measuring the antioxidant activity of plant extracts. In the DPPH assay, an antioxidant scavenges the free radicals and is used to measure the capacity of extracts to scavenge the stable radical DPPH formed in solution by donation of hydrogen atom or an electron (Mishra *et al.* 2012). On MeJA treatment, antioxidant activity in extract prepared from leaves and root tissues was increased, whereas there was no significant change in antioxidant activity of stem extracts. Among the three types of tissues tested, root extract showed the highest antioxidant activity. The $IC_{50}$ values for methanolic extracts of leaves, stem and roots of control and MeJA-treated plants are given in table 2. The antioxidant activity of all the extracts was lower than that of ascorbic acid ($6.04 \pm 0.32$), which was used as positive control.

### *Posttranscriptional regulation of CfCHS*

CHS catalyzes the first committed step of flavonoid biosynthesis, which is an important regulatory branch point, channelling flux of phenylpropanoid pathway towards biosynthesis of flavonoids. Several species of *Coleus* including *C. forskohlii* are known to produce rosmarinic acid. Rosmarinic acid also utilizes *p*-coumaroyl-CoA as a precursor. Downregulating *CfCHS* could help in diverting the metabolic flux towards synthesis of alternate compounds of commercial value such as rosmarinic acid or other phenylpropene phytochemicals. For instance, downregulating the strawberry CHS resulted in enhanced production of phenylpropene aroma compounds like eugenol (Hoffmann *et al.* 2011). As a result, we carried out an *in silico* search to find the miRNAs that may target *CfCHS*, thereby downregulating its expression.

MiRNAs are small noncoding RNAs that regulate the target mRNA levels, by cleavage of target or by suppressing its translation. They are known to play fundamental roles in various biological processes, including development and physiology (Bartel 2004; Sunkar *et al.* 2007). Our *in silico* search for mRNAs that may regulate *CfCHS* expression, identified four miRNAs targeting *CfCHS*, whose target

**Table 2.** Measurement of total flavonoid content and antioxidant activity in extracts prepared from leaves, stem and roots of *C. forskohlii* in response to MeJA treatment.

| | Total flavonoid content (mg quercitin equivalent (mg QAE) per gram dry weight of the plant material) | | DPPH radical scavenging activity ($IC_{50}$) | |
|---|---|---|---|---|
| | Control plant | MeJA-treated plant | Control plant | MeJA-treated plant |
| Leaves | 27.53 ± 2.68 | 34.85 ± 2.01 | 110.01 ± 6.10 | 81.75 ± 8.59 |
| Stems | 9.77 ± 0.88 | 9.79 ± 0.14 | 110.63 ± 3.35 | 113.74 ± 3.97 |
| Roots | 10.44 ± 0.79 | 22.48 ± 0.37 | 81.45 ± 6.99 | 61.95 ± 6.48 |

$P$ value $< 0.05$.
$IC_{50}$, the concentration of extracts ($\mu$g/mL) causing 50% inhibition of DPPH radical.

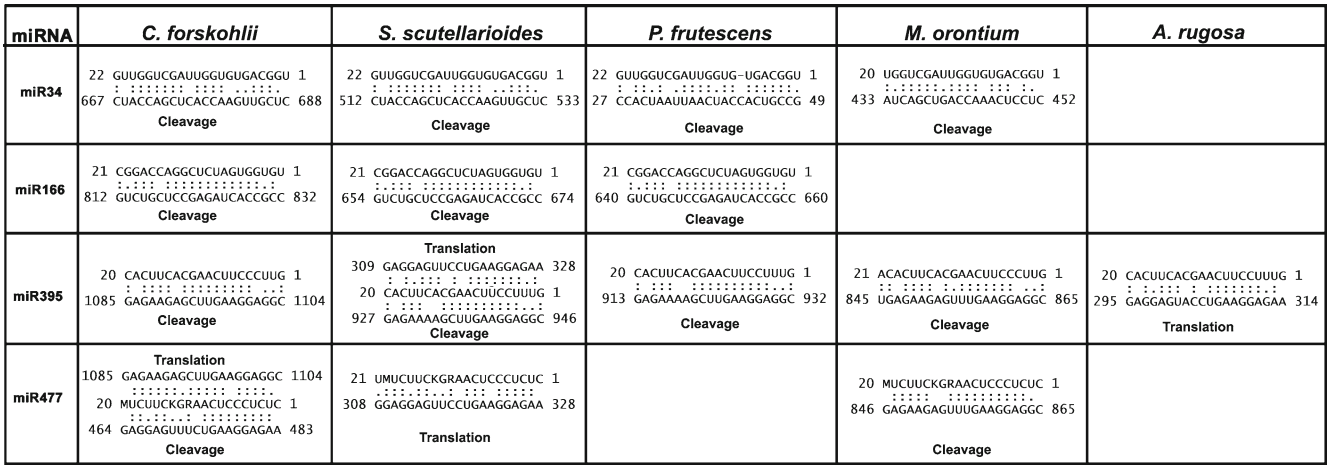


**Figure 9.** Prediction of miRNAs targeting CfCHS. Picture shows conservation of target sites of predicted miRNAs in CfCHS and related homologues from other plant species and their possible modes of action (cleavage or inhibition of translation).

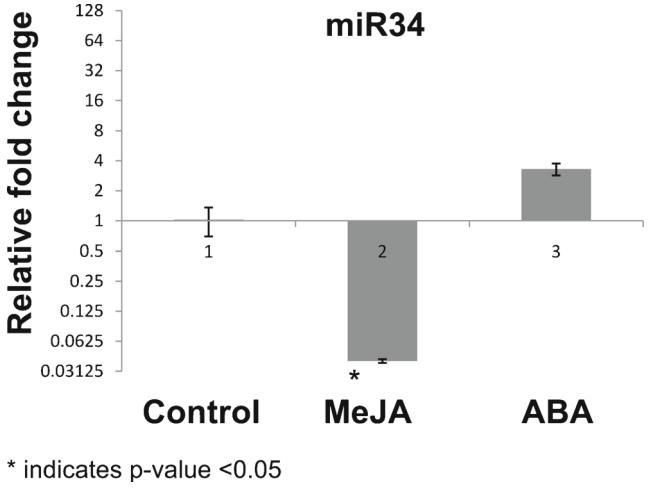


**Figure 10.** Expression profile of miR34, in response to elicitor: MeJA and ABA. 5.8S gene was used as housekeeping control. Expression of miR34 in 0.1% ethanol-treated (control) plants was used as baseline for calculating fold change. Three replicates were used for analysis. *$P < 0.05$.

sequence is conserved in at least three other homologues of *CHS* from four different plant species (*S. scutellarioides*, *A. rugosa*, *P. frutescens* and *M. orontium*) that were tested. MiR34, miR166 and miR395 were predicted to regulate *CfCHS* RNA levels through cleavage, while miR477 may act by translational repression as well as cleavage of target transcripts (figure 9). Earlier study in *Phelipanche ramosa* (L). Pomel (Orobanchaceae) reported that miR166 was downregulated in response to viroid infection (Ivanova *et al.* 2014). Flavonoids are already known to be involved in defense responses against pathogen infection (Jasinski *et al.* 2009; Päsold *et al.* 2010) and in a study, enhanced accumulation of *CHS* transcripts was reported in Norway spruce after pathogen infection (Nagy *et al.* 2004). Expression of miR166, miR395 and miR477 was undetectable in all treatments, whereas miR34 was downregulated and *CfCHS* was upregulated in MeJA-treated plants (figure 10), thus exhibiting an inverse correlation suggesting that miR34 is a potential regulator of CfCHS and flavonoid biosynthesis.

## Conclusion

Full-length clone of *CHS* gene was obtained from cDNA and genomic DNA of *C. forskohlii*. *CfCHS* showed dominant expression in young leaves and flowers. *CfCHS* was found to be significantly induced by MeJA with concomitant increase in total flavonoid content and antioxidant activity. Our results also indicate that miR34 is a potential posttranscriptional regulator of *CfCHS* expression and flavonoid biosynthesis.

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