Key genes in the phenylpropanoids biosynthesis pathway have different expression patterns under various abiotic stresses in the Iranian red and green cultivars of sweet basil (Ocimum basilicum L.)

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Abstract

Phenylpropanoids are a large and important family of plant secondary metabolites. The biosynthetic pathway of phenylpropanoids is induced in plants under environmental stresses to cope with these harmful conditions. In the present study, for the first time, we identified and characterized one reference gene (*ACTIN*) and three key biosynthetic genes (*4CL*, *C4H* and *CVOMT*) in the Iranian red and green cultivars of sweet basil. Also, the expression patterns of *4CL*, *C4H* and *CVOMT* biosynthetic genes were determined for the first time in the Iranian red and green cultivars of sweet basil under cold, drought, heat, light and salt stresses. The results showed that the *ACTIN*, *4CL*, *C4H* and *CVOMT* genes identified in the Iranian cultivars are identical to other cultivars in terms of all characteristics such as ORF length, protein sequence length, molecular weight, functional domains, lack of signal peptide, subcellular localization site, and secondary structures. Our results also revealed that the *4CL*, *C4H* and *CVOMT* biosynthetic genes have different expression in the Iranian red and green cultivars of sweet basil under abiotic stresses and their expression patterns are cultivar-dependent. The findings of this study can advance our knowledge of phenylpropanoids biosynthesis in plants under environmental stresses. These findings also can be used in plant breeding programs for stress tolerance in sweet basil and other plants.

Introduction

The medicinal plants produce a wide range of valuable therapeutic and industrial secondary metabolites (Saxena et al. 2013; Van Wyk and Wink 2018). Due to advantages such as easy availability, safety and cost-effectiveness, the medicinal plants have been widely used in various industries (Lim 2012; Saxena et al. 2013; Van Wyk and Wink 2018). Among all medicinal plants, the members of genus *Ocimum* are very valuable for their therapeutic compounds and they contain various secondary metabolites with medicinal properties (Miraj and Kiani 2016; Purushothaman et al. 2018). On the other hand, among 150 species of the genus *Ocimum*, secondary metabolites of sweet basil (*Ocimum basilicum* L.) are the most widely used in the pharmaceutical and cosmetics industries and this plant is the major essential oil-producing crop which is cultivated commercially all over the world (Nerio et al. 2010; Bilal et al. 2012; Miraj and Kiani 2016; Purushothaman et al. 2018). Sweet basil is an annual, herbaceous, aromatic medicinal plant of the Lamiaceae family (Khair-ul-Bariyah et al. 2012; Miraj and Kiani 2016).

The sweet basil contains chemical compounds such as terpenes, tannins, flavonoids and phenylpropanoids (Grayer et al. 1996; Bilal et al. 2012). Two phenylpropanoids, methyl eugenol and methyl chavicol, are the most important ingredients in sweet basil essential oil (Simon et al. 1990; Joshi 2014; Tahsili et al. 2012). The phenylpropanoids are produced from phenylalanine and tyrosine through shikimate pathway. Several enzymes are involved in phenylpropanoids biosynthesis from phenylalanine in plants (Herrmann and Weaver 1999; Haslam 2014). The phenylpropanoids biosynthetic pathway in plants begins with phenylalanine, which is then deaminated to cinnamic acid by phenylalanine amonia-lyase (*PAL*) enzyme. The *PAL* enzyme by converting phenylalanine to cinnamic acid plays a vital role in phenylpropanoids biosynthesis in plants (Achnine et al. 2004; Swedan 2013; Khakdan et al. 2018). The second key enzyme in phenylpropanoids biosynthesis, cinnamate 4-hydroxylase (*C4H*), converts

cinnamic acid to p-coumaric acid (Li et al. 2019). The *C4H* acts via an enzyme complex and connects phenylpropanoid biosynthetisis to other metabolic processes (Winkel-Shirley 1999; Achnine et al. 2004). A small family of *C4H* genes exists in some plant such as *Zea mays* and *Medicago sativa*, whereas, other plants such as *Brassicanapus*, *Pisum sativum*, *Arabidopsis thaliana* and *Petroselinum crispum* contain only one *C4H* gene (Frank et al. 1996; Koopmann et al. 1999; Chen et al. 2007; Andersen et al. 2008; Xu et al. 2009; Douglas et al. 2011). Over-expression of *C4H* enhanced decursinol angelate level in hairy roots of *Angelica gigas* (Park et al. 2012). The antisense suppression of *C4H* gene reduced levels of chlorogenic acid, flavonoids and lignins in *M. sativa* transgenic plants (Reddy et al. 2005). Members of 4-coumarate-CoA ligase (*4CL*) gene family catalyze the conversion of 4-coumaric acid to 4-coumaroyl-CoA and participate in phenylpropanoid biosynthesis in plants (Hu et al. 1998; Hamberger and Hahlbrock 2004; Wang et al. 2016). Members of *4CL* gene family are also necessary in production of 4-cinnamic acid CoA esters that are required for the formation of plant products such as lignin, stilbenes and flavonoids (Wang et al. 2016). Over-expression of *4CL* gene enhanced flavone level in hairy roots of *Scutellariabaicalensis* (Kim et al. 2014). On the other hand, down-regulation of *4CL* reduced lignin content in transgenic switchgrass (Xu et al. 2011). The chavicol O-methyltransferase (*CVOMT*) transfers a methyl group from S-adenosylmethionine to chavicol and produces methylchavicol (Gang et al. 2002). The *CVOMT* has higher expression level in young leaves of basil compared to older leaves (Gang et al. 2002). Different chemotypes the red and green sweet basil contain various phenylpropanoid content and some of them are rich in specific phenylpropanoids (Beatovic et al. 2015; Bilal et al. 2012; Simon et al. 1990).

Previous studies have reported that phenylpropanoids biosynthesis in plants is affected by growth and development, biotic and abiotic stresses (Batard et al. 1997; Solecka 1997; Bellés et al. 2008; Silva et al. 2012; Niinemets et al. 2013; Meek 2015; Deng and Lu 2017; Rastogi et al. 2019). In a study, the insect and mechanical induced stresses enhanced production of phenylpropanoids such as eugenol, eugenol acetate, dillapiole and myristicin in the leaves of *Mangiferaindica* (Silva et al. 2012). In the other study, the cold, drought, flood and salt stresses changed expression of phenylpropanoids biosynthetic genes and also altered accumulation level of these compounds in *Ocimumtenuiflorum* (Rastogi et al. 2019). In the similar study, water stress increased expression of genes involved in phenylpropanoids biosynthesis such as *PAL*, *C3H*, *4CL* in *Oryza sativa* (Yang et al. 2006; Moura et al. 2010). Similar results were also observed in *Citrullus lanatus* plant under drought stress (Yoshimura et al. 2008; Moura et al. 2010).

Because of the effect of environmental stresses on expression of phenylpropanoids biosynthetic genes and phenylpropanoid content, the current study was conducted to investigate the expression of three key biosynthetic genes in the Iranian red and green cultivars of sweet basil under five environmental stresses. In the present study, for the first time we evaluated the effect of cold, drought, heat, light and salt stresses on expression of *4CL*, *C4H* and *CVOMT* genes in the Iranian red and green cultivars of sweet basil.

Materials And Methods

Plant Materials, Stress Treatments and Sampling

Seeds of the Iranian red and green cultivars of sweet basil were provided from seed and plant improvement institute of Iran. Seeds were surface sterilized by 5% (v/v) sodium hypochlorite and sown plastic pots containing sterilized soil mixture (40% clay and 60% peats). Pots were fed with NPK fertilizer (20:20:20) and kept at 20/20^{o}C (light/dark) with a photoperiod of 16/8 h. The regular irrigation of pots was performed to maintain normal soil moisture level (Chang et al. 2005). Five weeks plants (7-8 leaves plants) were used to stress treatment. For cold stress, plants were kept in 10^{o}C for 24 h and then transferred to 8^{o}C for 24 h. For heat stress, pots containing five weeks plants were kept in 30^{o}C for 48 h. For drought stress, the field capacity (FC) of pots was determined using the gravimetric approach (Belhassen 1997) and pots containing five weeks plants were kept in 50% FC for one week. For light stress, pots were transferred to dark and kept them in 16^{o}C for 48 h. Finally, for salt stress, pots were irrigated with 100mM NaCl for 48 h. The control plants were well irrigated and kept in normal condition (20/20^{o}C light/dark and photoperiod of 16/8 h) until subsequent analyses. Leaves of control and stressed plants were harvested and immediately frozen in liquid N2. Frozen leaf tissues were transferred to -80°C and kept for subsequent analyses.

RNA Isolation and cDNA Synthesis for real-time PCR

Frozen leaf tissues of control and stressed plants were thoroughly ground in liquid N2 using mortar and pestle. Total RNA was isolated from ground tissues using Yekta Tajhiz Azma RNA isolation kit (Tehran, Iran) according to the manufacturer's instructions. The integrity of isolated total RNA was evaluated using spectrophotometer and agarose gel electrophoresis. Removing of genomic DNA from isolated total RNA was performed using Yekta Tajhiz Azma DNase I kit (Tehran, Iran) according to the manufacturer's instructions. Two micrograms of DNase I-treated total RNA was used for first strand cDNA synthesis. The cDNA was synthesized using Yekta Tajhiz Azma cDNA synthesis kit (Tehran, Iran) according to the manufacturer's instructions. The complete synthesis of first strand of cDNA and DNA removal from total RNA and was confirmed by PCR using specific *ACTIN* primers (Table 1).

Molecular Cloning of Biosynthetic Genes and Design of Primers

To identify coding sequences of *O. basilicum* biosynthetic genes, the protein sequences of known *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes were retrieved from the NCBI protein databse. The protein sequences of each gene were separately aligned against the EST library of *O. basilicum* using NCBI tBLASTn tool (Johnson et al. 2008). The resulting ESTs for each gene were separately pooled and assembled in CLC Genomics Workbench 9.0 software (Qiagen, Valencia, USA). The resulting contigs were compared with the GenBank nr/nt database using BLASTn tool and the contigs with similarity to known *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes were selected for further analyses. The selected contigs were evaluated for the presence of complete open reading frames (ORFs) of *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes. The complete ORFs of *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes were used to design primers by Allele ID 6.0 software (Table 1). The complete ORFs of *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes were amplified by PCR. PCR was performed using gene specific primers and *Pfu* DNA polymerase (Thermo Fisher Scientific, Lenexa, USA) on cDNA template. The PCR products were tailed using *Taq* DNA polymerase (SinaClon BioScience,

Tehran, Iran) and cloned into pTZ57R/T plasmid (Thermo Fisher Scientific, Lenexa, USA). The plasmids containing *ACTIN*, *4CL*, *C4H*, and *CVOMT* gene fragments were sequenced. The results of sequencing were analyzed in CLC Genomics Workbench 9.0 software (Qiagen, Valencia, USA) and compared to GenBank using BLASTn tool. The specific primers for real-time PCR were designed based on the sequence of identified genes. The complete coding sequences of identified genes were inserted to Allele ID 7.0 software and specific primers were designed (Table 1). The specificity of the designed primers was evaluated by PCR (sweet basil genomic DNA as template) and NCBI Primer BLAST tool.

In silico and phylogenetic *analyses*

In silico analyses of identified genes were performed using ORF finder, CDD, Interproscan, Pfam, Expasy ProtParam , CELLO2GO, SignalP, Phyre2, Vector NTI 10.3, and CLC Genomics Workbench 9.0 tools (Gasteiger et al. 2005; McGuffin et al. 2000; Yu et al. 2014; Marchler-Bauer et al. 2014; Finn et al. 2016; Jones et al. 2014; Petersen et al. 2011; Kelley et al. 2015). Multiple sequence alignment (MSA) of protein sequences of *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes was performed using Vector NTI 10.3.

Gene expression analysis by Real-time PCR

To minimize sampling and pipetting errors, the cDNAs were diluted in distillated water. Five microliters of the diluted cDNAs (1:10) was used as template in the real-time PCR. Real-time PCR was performed using Yekta Tajhiz Azma SYBR Premix (Tehran, Iran) on a Bio-rad thermocycler. The specificity of the amplifications was evaluated by melting curve analysis and agarose gel electrophoresis. All amplification reactions were performed in three biological and three technical replications. The efficiency, reproducibility and dynamic range of amplification reactions were evaluated using a standard curve. The identified *ACTIN* gene was used as reference gene. The resulted cycle thresholds (C_t) of all genes were normalized by the C_t values of the *ACTIN* gene. The relative expression of *4CL*, *C4H* and *CVOMT* genes were determined using the $2^{\Delta\Delta C}$t method (Livak and Schmittgen 2001). The Mean of C_t values were calculated across biological and technical replicates. Statistical analysis was performed using MINITAB 14 software. The Tukey's test was used for multiple comparisons of means at $P < 0.05$.

Results

Characterization of the *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes

The PCR amplified fragments of *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes and confirmed the results of EST assembly. The gel electrophoresis showed products with size of 1,334, 1,815, 1,214, and 1631 bp for *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes, respectively. The results of sequencing showed complete ORFs with size of 1,134, 1,704, 1,071, and 1,518 bp for *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes, respectively (Table 2) (Supplementary file). The complete ORFs of *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes began with ATG as start codon and ended with TAA and TGA as stop codons (Supplementary file). The *ACTIN*, *4CL*, *C4H*, and *CVOMT* genes were completely identical in red and green cultivars. For this reason, only one sequence

was included in the analyses. The identified *ACTIN* gene showed 89.07% identity to *Agastache rugosa*, 88.27% identity to *Sesamum indicum*, 87.92% identity to *Erythranthe lewisii*, 88.22% identity to *Leucas cephalotes* and 86.64% identity *Erythranthe guttatus*. The nucleotide sequence of *4CL* gene identified from Iranian cultivars of sweet basil showed 100% identity to *O. basilicum*, 92.9% identity to *O. tenuiflorum*, 85.34% identity to *Agastacherugosa*, 83.9% identity to *Salviamiltiorrhiza* and 81.72% identity to *Scutellariabaicalensis*. The nucleotide sequence of *C4H* gene displayed 100% identity to *O. basilicum*, 94.66% identity to *O. tenuiflorum*, 87.94% identity to *S. miltiorrhiza*, 86.89% identity to *Mentha spicata* and 86.36% identity to *Mentha aquatica*. The identified *CVOMT* gene exhibited 100% identity to *O. basilicum*, 98.71% identity to *Ocimum americanum*, 96.22% identity to *Ocimum africanum*, 96.12% identity to *Ocimum kilimandscharicum* and 91.67% identity *O. tenuiflorum*. These results were similar in two Iranian red and green cultivars of sweet basil.

In frame translation of complete ORFs produced proteins with length of 377, 567, 356, 505 and amino acids for *ACTIN*, *4CL*, *CVOMT*, and *C4H* genes, respectively (Table 2). The molecular weight of *ACTIN*, *4CL*, *CVOMT*, and *C4H* proteins were computed 41.664, 61.12, 39.922, and 58.044 kDa (Table 2).

The signal peptide prediction showed no signal sequence in the structure of *ACTIN*, *4CL*, *C4H*, and *CVOMT* proteins (Table 3) (Supplementary file). The subcellular localization prediction exhibited that all identified proteins are localized in the cytoplasm (Table 3) (Supplementary file). The secondary structure prediction revealed the presence of alpha helices and beta strands with variable ratios in all identified proteins (Table 3). The search for functional domain showed the presence of NBD_sugar-kinase_HSP70 domain in *ACTIN*, 4-coumarate-CoA ligase domain in *4CL*, tans-cinnamate 4-monooxygenase domain in *C4H*, and AdoMet-MTase Dimerization domains in *CVOMT* structure (Table 3) (Supplementary file).

The protein sequence of *O. basilicumACTIN* exhibited 97-99% identitiy to its orthologs in other mono- and dicotyledon plant species (Figure 1). The *O. basilicumACTIN* showed the highest and lowest identity with *Sesamumindicum* (99%) and *Z. mays* (67%), respectively (Figure 1).

The MSA results showed that the *O. basilicum4CL* has 67-86% identitiy to its orthologs in other mono- and dicotyledon plant species (Figure 2). The *O. basilicum4CL* gene showed the highest and lowest identity with *Sesamumindicum* (86%) and *Triticumaestivum* (67%), respectively (Figure 2).

The MSA results showed that the *O. basilicumC4H* has 85-92% identitiy to its orthologs in other mono- and dicotyledon plant species (Figure 3). The *O. basilicumC4H* gene showed the highest and lowest identity with *Striga asiatica* (92%) and *Sorghum bicolor* (85%), respectively (Figure 3).

The protein sequence of *O. basilicumCVOMT* exhibited 42-60% identitiy to its orthologs in other mono- and dicotyledon plant species (Figure 4). The *O. basilicumCVOMT* showed the highest and lowest identity with *Catalpa bungei* (60%) and *Asparagusofficinalis* (42%), respectively (Figure 4).

Gene expression analysis

The results of relative expression of *4CL*, *C4H*, and *CVOMT* genes in red and green cultivars of basil under different environmental stresses treatments have been shown in the figures 1, 2 and 3. Analysis of variance revealed significant differences in the expression of *4CL* gene under different stresses in both red and green cultivars of basil. In the cold, heat and light stresses similar significant changes in expression of *4CL* gene were observed in both red and green cultivars (Figure 5). The expression of *4CL* gene was significantly altered in green cultivar under drought and salt stresses, while, in the red cultivar, no significant changes were observed in expression of this gene (Figure 5). The expression level of *4CL* gene was significantly decreased under cold, heat and light stresses in both red and green cultivars (Figure 5). In drought and salt stresses, the expression level of *4CL* gene was significantly decreased in green cultivar, while, it remained unchanged in the red cultivar (Figure 5). Comparison of means showed that the changes in the expression level of *4CL* gene under different stresses were similar in both red and green cultivars except in the red cultivar exposed to salt stress (Figure 5).

The results of analysis of variance showed significant changes in the expression of *C4H* gene under different stresses in both red and green cultivars of basil. In the salt and light stresses a similar significant changes in expression of *C4H* gene was observed in both red and green cultivars (Figure 6). On the other hand, in the drought and heat stresses a different significant change in expression of *C4H* gene was observed in both red and green cultivars (Figure 6). The expression of *C4H* gene was significantly altered in red cultivar under cold stress, while, in the green cultivar, no significant changes were observed in expression of this gene (Figure 6). The expression level of *C4H* gene was significantly decreased under salt and light stresses in both red and green cultivars (Figure 6).

Analysis of variance showed significant differences in the expression of *CVOMT* gene under different stresses in both red and green cultivars of basil. The expression of *CVOMT* gene significantly increased under the drought stress in both red and green cultivars (Figure 7). In the salt stress, the expression of *CVOMT* gene was significantly decreased in the green cultivar, but it remained unchanged in the red cultivar (Figure 7). In the cold stress, the expression of *CVOMT* gene was significantly increased in the green cultivar and decreased in the red cultivar (Figure 7). In heat and light stresses, the expression level of *CVOMT* gene was significantly decreased in both red and green cultivars. This reduction was the same in both cultivars under the light stress, but in heat stress, the green cultivar showed a much greater reduction in the expression of *CVOMT* gene (Figure 7).

Discussion

Among all species of the genus *Ocimum*, secondary metabolites of *O. basilicum* (sweet basil) are the most widely used in the pharmaceutical and cosmetics industries (Nerio et al. 2010; Bilal et al. 2012; Miraj and Kiani 2016; Purushothaman et al. 2018). In the present study, we investigated the effect of abiotic stresses on the expression of *4CL*, *C4H*, and *CVOMT* biosynthetic genes in the Iranian red and green cultivars of sweet basil. For this purpose, we first identified and characterized *4CL*, *C4H*, and *CVOMT* biosynthetic genes and *ACTIN* reference gene in the tested cultivars. In the next step, we

examined the expression of these genes under the cold, drought, heat, light and salt stresses in the leaves of these cultivars.

The *ACTIN* genes encode the essential multi-functional proteins that found in all eukaryotic organisms. The *ACTIN* plays important role in many important cellular processes, including cell division, signaling, cytokinesis, cell motility, and organelle movement (Dominguez and Holmes 2011; Gunning et al. 2015). The *ACTIN* gene has been identified from different plant species. The *ACTIN* genes identified from the Iranian red and green cultivars of basil did not show any difference with other basil cultivars. Our results showed that the *ACTIN* genes identified in Iranian cultivars were identical to other cultivars in terms of all characteristics such as ORF length, protein sequence length, molecular weight, functional domains, lack of signal peptide, subcellular localization site, and secondary structures. Due to the optimized structure, the protein sequence of *ACTIN* has been highly conserved during evolution (Dominguez and Holmes 2011; Gunning et al. 2015).

The *4CL* gene catalyzes the conversion of 4-coumaric acid to 4-coumaroyl-CoA and participates in phenylpropanoid biosynthesis in plants (Hu et al. 1998; Hamberger and Hahlbrock 2004; Wang et al. 2016). The *4CL* gene has been identified from different plant species. The *4CL* genes identified from the Iranian red and green cultivars of basil did not show any difference with other basil cultivars. Our results showed that the *4CL* genes identified in Iranian cultivars were identical to other cultivars in terms of all characteristics such as ORF length, protein sequence length, molecular weight, functional domains, lack of signal peptide, subcellular localization site, and secondary structures.

Numerous studies reported that the expression of *4CL* gene involved in biosynthesis of phenylpropanoid decreased under abiotic stresses in plants (Lavhale et al. 2018). The expression of *4CL* gene decreased under wounding stress in *A. thaliana* (Soltani et al. 2006). The expression of *4CL* gene decreased in *O. basilicum* in response to drought stress (Mandoulakani et al. 2017). Our results were consistent with the previous studies. The expression of *4CL* gene significantly decreased in green cultivar under cold, drought, heat, light, and salt stresses. The expression of this gene also significantly decreased in the red cultivar under cold, heat and light stresses and it remained unchanged under drought and salinity stresses. In the heat stress, the expression of *4CL* gene in green cultivar showed a greater decrease compared to red cultivar. These results indicate that the expression of *4CL* gene is not similar in different cultivars of basil and it depends on the cultivar.

The *C4H* gene is a key enzyme in phenylpropanoids biosynthesis that catalyzes conversion of cinnamic acid to p-coumaric acid (Li et al. 2019). So far, the *C4H* gene have been identified in *Z. mays*, *M. sativa*, *B. napus*, *P. sativum*, *A. thaliana* and *P. crispum* plants (Frank et al. 1996; Koopmann et al. 1999; Chen et al. 2007; Andersen et al. 2008; Xu et al. 2009; Douglas et al. 2011). Our results demonstrated that the *C4H* genes identified from the Iranian red and green cultivars of basil were identical to other cultivars in terms of all characteristics such as ORF length, protein sequence length, molecular weight, functional domains, lack of signal peptide, subcellular localization site, and secondary structures. These genes did not show any difference with other basil cultivars.

Several studies have reported that the expression of *C4H* gene in plants changes under abiotic stresses (Sharma et al. 2019). The wounding, cold and salt stresses increased the expression of *C4H* gene in the stem tissues of *Hibiscuscannabinus* L. (Kim et al. 2013). The expression of *C4H* gene remained unchanged in the cold-stressed leaf tissues of *O. basilicum* var. keshkeni luvelou (Rezaie et al. 2020). The *C4H* gene highly expressed under the wounding and salt stresses in the *Carthamustinctorius* (Sadeghi et al. 2013). The copper stress increased the expression of *C4H* gene in the *Vitis vinifera* (Leng et al. 2015). The expression of *C4H* gene increased under the drought stress in the *Fragariaananassa* and Lotus japonicas plants (Perin et al. 2019; García-Calderón et al. 2015). The salt stress increased the expression of *C4H* gene in the *F. ananassa* and Olea europaea plants (Perin et al. 2019; Rossi et al. 2016). The expression of *C4H* gene increased under the chilling stress in the *Prunuspersica* (Wang et al. 2019).

Our results indicated that the expression of *C4H* gene show different pattern under the drought, cold, and heat stresses in the red and green cultivars. The expression of *C4H* gene significantly increased in green cultivar under the drought stress, while it significantly decreased in red cultivar. In the cold stress, the expression of *C4H* gene significantly increased in red cultivar and it remained unchanged in green cultivar. The expression of *C4H* gene significantly increased in red cultivar under the heat stress, while it significantly decreased in green cultivar. The salt and light stresses significantly decreased the expression of *C4H* gene in red and green cultivars. In the light stress, the expression of *C4H* gene in green cultivar showed a greater decrease compared to red cultivar. These results indicate that the expression of *C4H* gene is not similar in different cultivars of basil and it depends on the cultivar.

The *CVOMT* catalyzes the conversion of chavicol to methylchavicol (Gang et al. 2002). The *CVOMT* genes identified from the Iranian red and green cultivars of basil did not show any difference with other basil cultivars. Our results showed that the *CVOMT* genes identified in Iranian cultivars were identical to other cultivars in terms of all characteristics such as ORF length, protein sequence length, molecular weight, functional domains, lack of signal peptide, subcellular localization site, and secondary structures.

The previous studies have shown that the expression of *CVOMT* gene in *O. basilicum* increases under drought stresses (Mandoulakani et al. 2017; Lavhale et al. 2018; Khakdan et al. 2017).

The expression of *CVOMT* gene significantly decreased in the cold-stressed leaf tissues of O. basilicum var. keshkeni luvelou (Rezaie et al. 2020). In case of drought stress, our results were consistent with the previous studies. The expression of *CVOMT* gene significantly increased under the drought stress in both green and red cultivars. In the cold stress, the expression of *CVOMT* gene significantly increased in green cultivar, while it significantly decreased in red cultivar. The expression of *CVOMT* gene significantly decreased under salt stress in green cultivar and it remained unchanged in red cultivar. In the heat stress, the expression of *CVOMT* gene in green cultivar showed a greater decrease compared to red cultivar. The light stress significantly decreased the expression of *CVOMT* gene in both red and green cultivars. These results indicate that the expression of *CVOMT* gene is not similar in different cultivars of basil and it depends on the cultivar.

The abiotic environmental stresses cause damage to plant processes, including growth and development, metabolism, photosynthesis and nutrition by inducing the production of free radicals (Sharma et al. 2019; Huang et al. 2019). Thus, plants activate protective systems, including radical scavenging processes and antioxidant compounds (such as phenylpropanoids and other phenolic compounds) to cope with stress condition (Jenks and Hasegawa 2005; Pereira 2016; Korkina 2007). Phenolic compounds act as strong radical scavenging agents due to their ability to donate electrons and hydrogen atoms (Huang et al. 2019; Sharma et al. 2019).

Previous studies have reported that different cultivars of a plant species produce different ratios of the phenolic compounds in response to environmental stresses. The different cultivars of *V. vinifera* produced different composition of phenolic compounds under drought stress (Pinasseau et al. 2017). The results of other study indicated that the phenylpropanoids accumulate at different levels in the UV-sensitive and UV-resistant cultivars of *Vacciniumcorymbosum* (Escobar et al. 2017). In the similar study, the water stress had different effects on phenylpropanoids content and on expression of their biosynthetic genes of *O. basilicum* cultivars (Khakdan et al. 2017). In the study of Rossi et al. (2016), the salt-tolerant and salt-sensitive cultivars of *O. europaea* accumulated different levels of phenylpropanoid under salt stress (Rossi et al. 2016).

Different expression pattern of phenylpropanoids biosynthetic genes under environmental stresses in different cultivars of basil can be caused by structural and morphological differences, differences in cell signaling processes, genetic and epigenetic differences. Cultivars with different morphology and structure show different levels of stress resistance and also display different responses to stress. The cell signaling processes are closely related to plant morphology and differences in morphology affect signaling processes. Differences in gene expression regulation, the presence or absence of specific regulatory cis-acting elements in the promoter of biosynthetic genes differences in the number of these elements are the most important genetic differences that may exist among cultivars. Distribution of particular cis-acting elements in the promoter of biosynthetic genes can change the gene expression pattern in different cultivars. Finally, epigenetic differences such as histone modifications and chromatin remodeling are other factors that can cause different responses in cultivars.

Conclusion

To our knowledge, the current study is the first report of the identification and characterization of three phenylpropanoids biosynthetic genes in the Iranian red and green cultivars of sweet basil. Also in our study, the expression patterns of *4CL*, *C4H* and *CVOMT* biosynthetic genes were determined for the first time in the Iranian red and green cultivars of sweet basil under cold, drought, heat, light and salt stresses. Our results showed that the *4CL*, *C4H* and *CVOMT* genes identified in Iranian cultivars are identical to other cultivars in terms of all characteristics such as ORF length, protein sequence length, molecular weight, functional domains, lack of signal peptide, subcellular localization site, and secondary structures. Our results also revealed that the expression pattern of *4CL*, *C4H* and *CVOMT* biosynthetic genes is cultivar-dependent. However, to better understand the differences among different sweet basil cultivars in

response to environmental stresses, the transcriptome of cultivars should be evaluated under stress condition.

The findings of the current study can advance our knowledge of phenylpropanoids biosynthesis in plants under environmental stresses. These findings also can be used in plant breeding programs for stress tolerance in sweet basil and other plants.

Declarations

Compliance with ethical standards

Conflict of interest: The authors have no conflicts of interest to declare.

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Tables

Table 1. Primers used for the molecular cloning and gene expression analysis.

Primer name	Sequence (5□"3□)	Product Length (bp)	Ta (°C)	
Full-*4CL*-F	CCTTCTTCCTCTTTCTTCTTC	1,815	60	Molecular Cloning
Full-*4CL*-R	ATGAACAAGGTAGGATAAATGG			
Full-*CVOMT*-F	AGTTCACCATATAAATTACATCCA	1,214	60	
Full-CVOMT-R	AAGTATTCGTTGCCTTTACATA			
Full-*C4H*-F	CACCGAAAATGGATCTTCTC	1,631	59	
Full-*C4H*-R	CCAATACCAACACTACAACAT			
Full-*ACTIN*-F	CTCTTCCGATCTCTCTCTTAA	1,334	61	
Full-*ACTIN*-R	CATCAGAAATCACTTCATAGACT			
4CL-F	CGACTGCGAGAACCATAG	100	53	Gene Expression Study
4CL-R	TCCTTCACCCTGTCTACG			
CVOMT-F	GGGAGAAGTTAGCAAATAAGC	163	52	
CVOMT-R	TTAGCCATCGTTCCATTACC			
C4H-F	AATCTCCACGACGCCAAG	151	57	
C4H-R	CGACTTTAGCCTCCTCTTCC			
ACTIN-F	GACAACATACAACTCCATCAT	126	58	
ACTIN-R	GTTATCTCCTTGCTCATTCTG			

F: Forward primer; R: Reverse primer; T_a: Annealing temperature

Table 2. The features of identified genes.

Gene Name	cDNA length (bp)	ORF length (bp)	Protein Length (aa)	MolecularWeight (kDa)
4CL	1,815	1,704	567	61.12
CVOMT	1,214	1,071	356	39.922
C4H	1,631	1,518	505	58.044
ACTIN	1,334	1,134	377	41.664

Table 3. Functional characterization of identified genes.

Gene Name	Signal sequence	Localization site	Secondary structures	Functional domain
4CL	No	Cytoplasmic	41% α and 21% β	4-coumarate-CoA ligase
CVOMT	No	Cytoplasmic	56% α and 13% β	AdoMet-MTase/ Dimerization domain
C4H	No	Cytoplasmic	64% α and 8% β	Tans-cinnamate 4-monooxygenase
ACTIN	No	Cytoplasmic	38% α and 23% β	NBD_sugar-kinase_HSP70

Figures

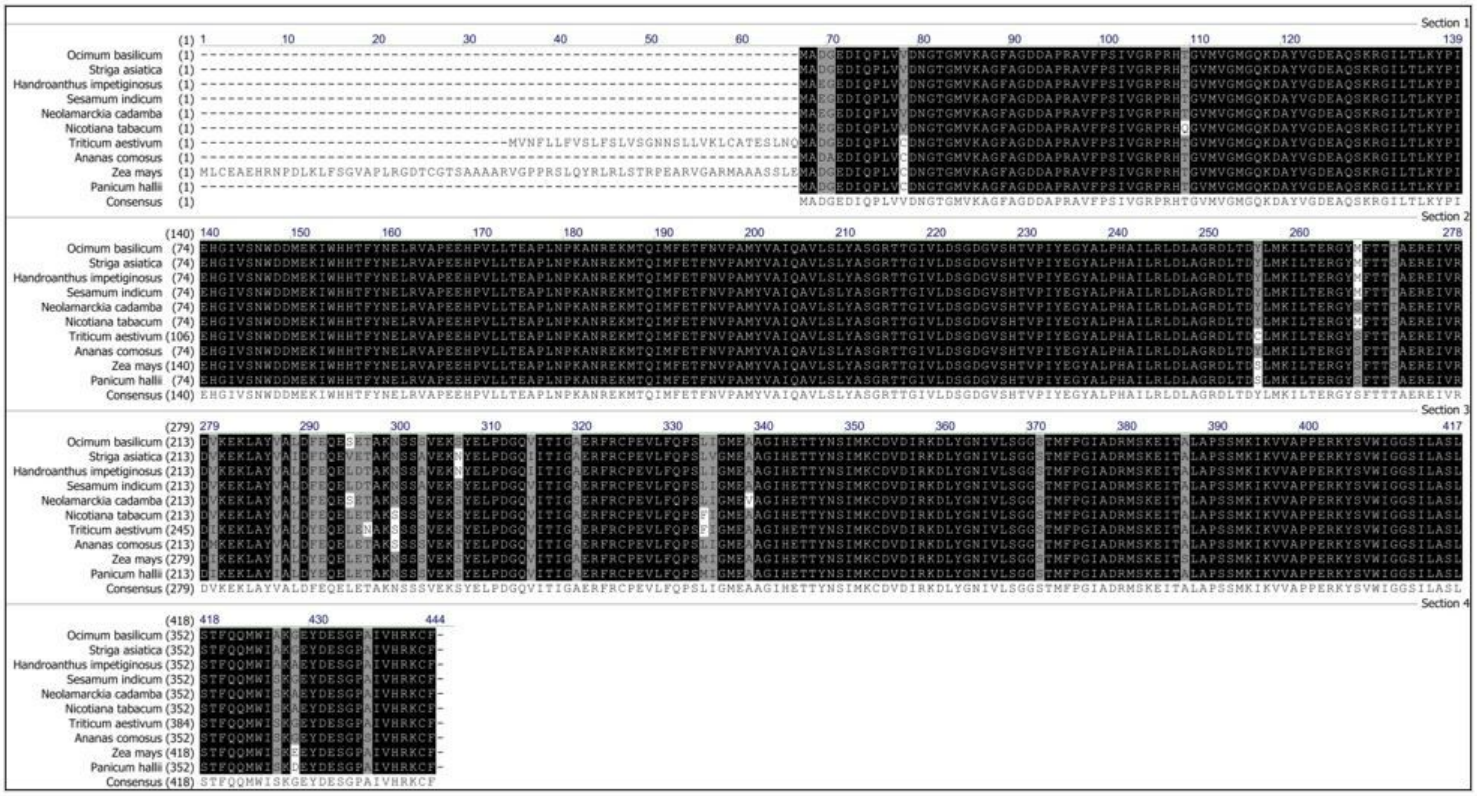

Figure 1

Multiple sequence alignment of identified ACTIN of O. basilicum with its orthologs in other mono- and dicotyledon plant species. The black and grey colors show identical and conservative amino acids, respectively. The numbers above the alignment display the position of amino acids. Gaps (-) were inserted to improve the alignment.

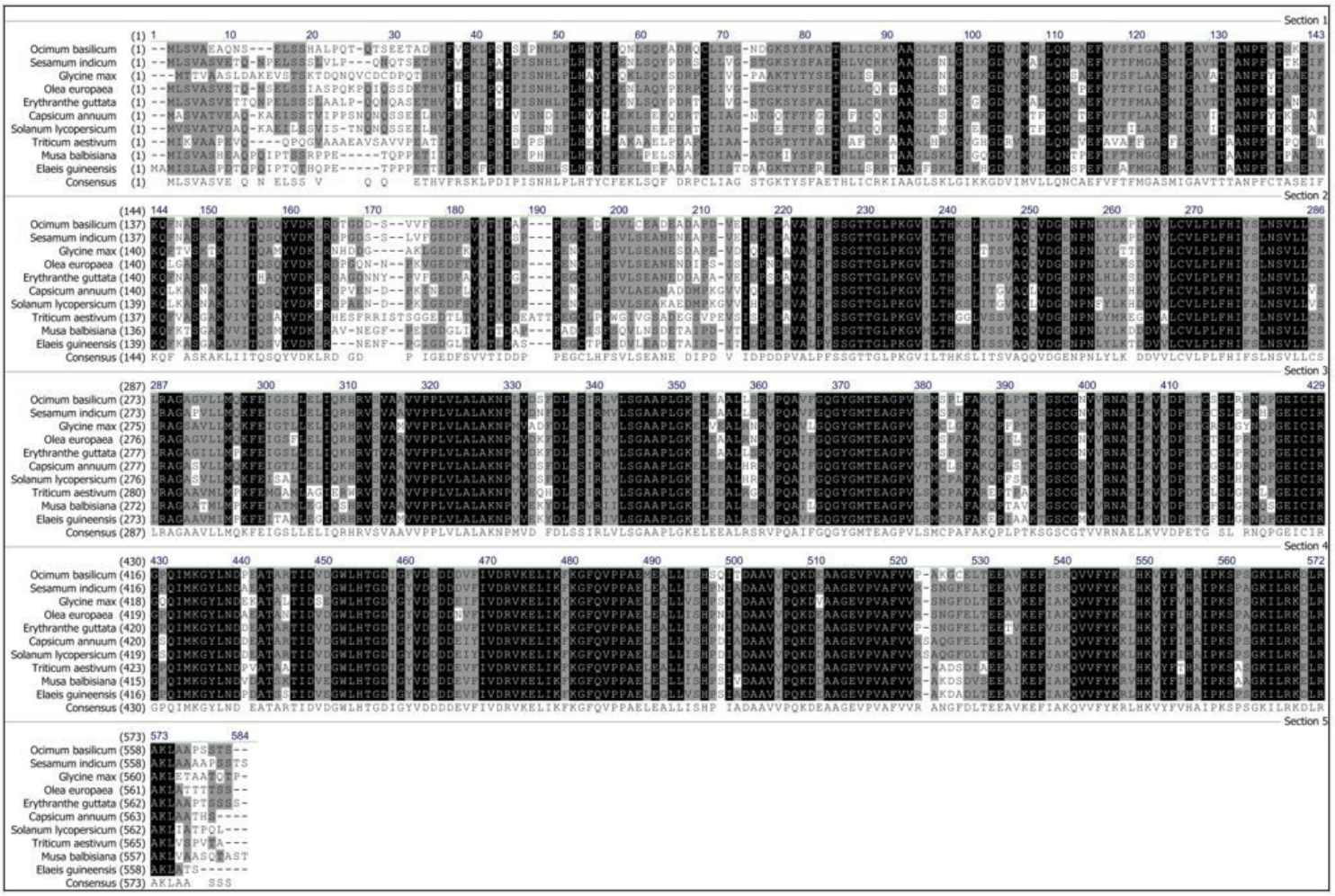

Figure 2

Multiple sequence alignment of identified 4CL of O. basilicum with its orthologs in other mono- and dicotyledon plant species. The black and grey colors show identical and conservative amino acids, respectively. The numbers above the alignment display the position of amino acids. Gaps (-) were inserted to improve the alignment.

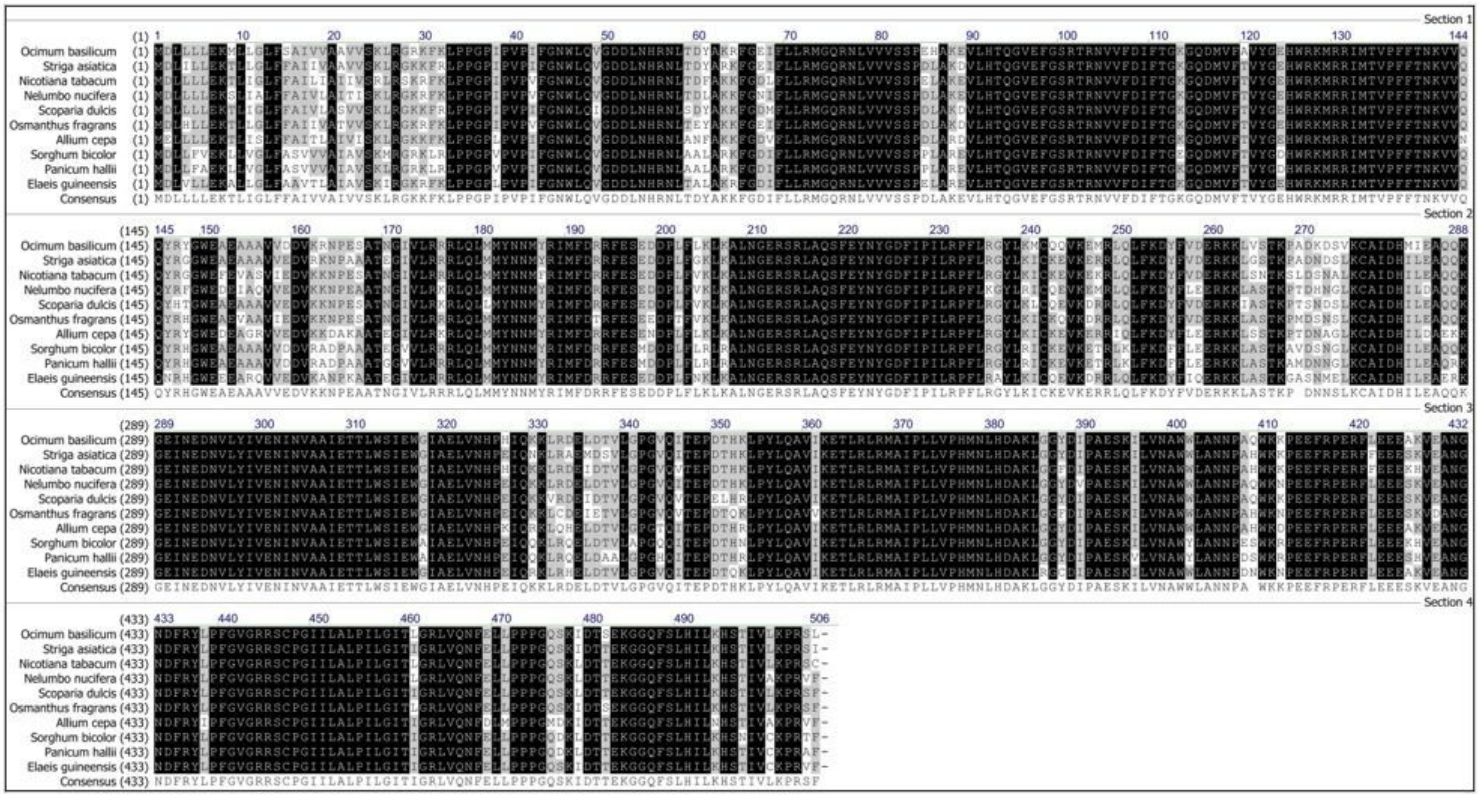

Figure 3

Multiple sequence alignment of identified C4H of O. basilicum with its orthologs in other mono- and dicotyledon plant species. The black and grey colors show identical and conservative amino acids, respectively. The numbers above the alignment display the position of amino acids.

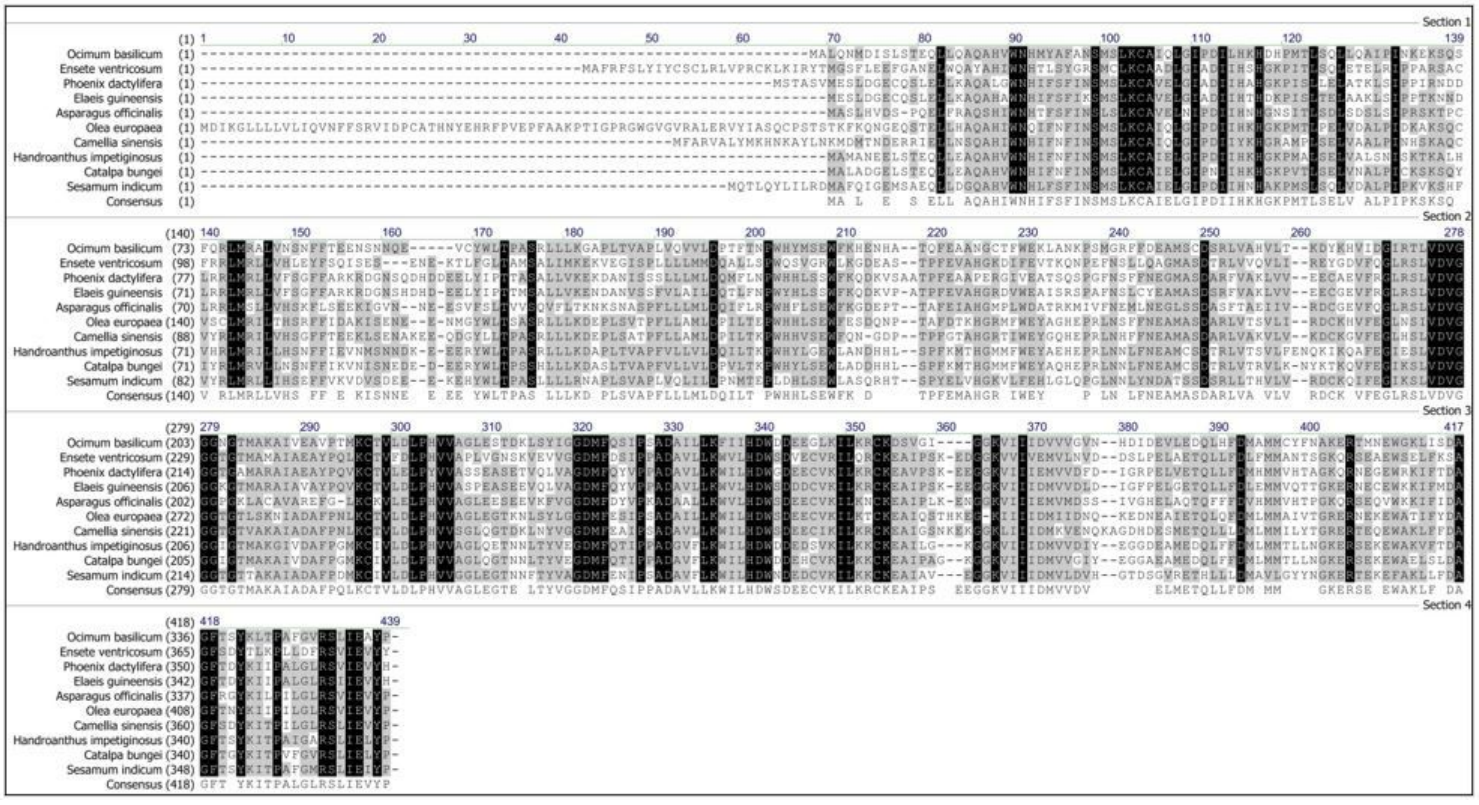

Figure 4

Multiple sequence alignment of identified CVOMT of O. basilicum with its orthologs in other mono- and dicotyledon plant species. The black and grey colors show identical and conservative amino acids, respectively. The numbers above the alignment display the position of amino acids. Gaps (-) were inserted to improve the alignment.

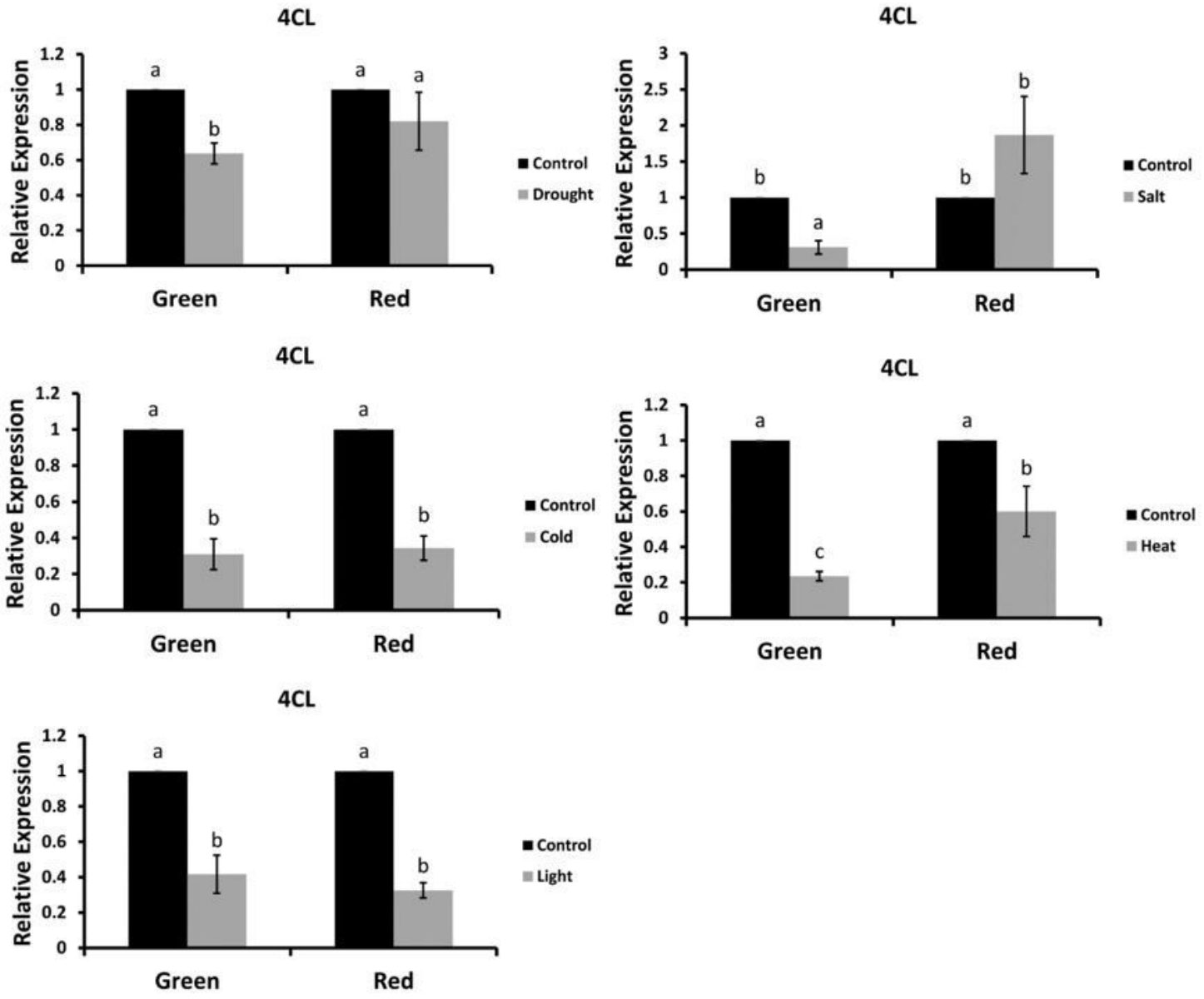

Figure 5

Expression pattern of 4CL gene in red and green cultivars of basil under different abiotic stresses. Bars show standard errors. Letters above the error show significant differences. Means with the same letter are not significantly different. The Tukey's test was used for multiple comparisons of means at $P < 0.05$.

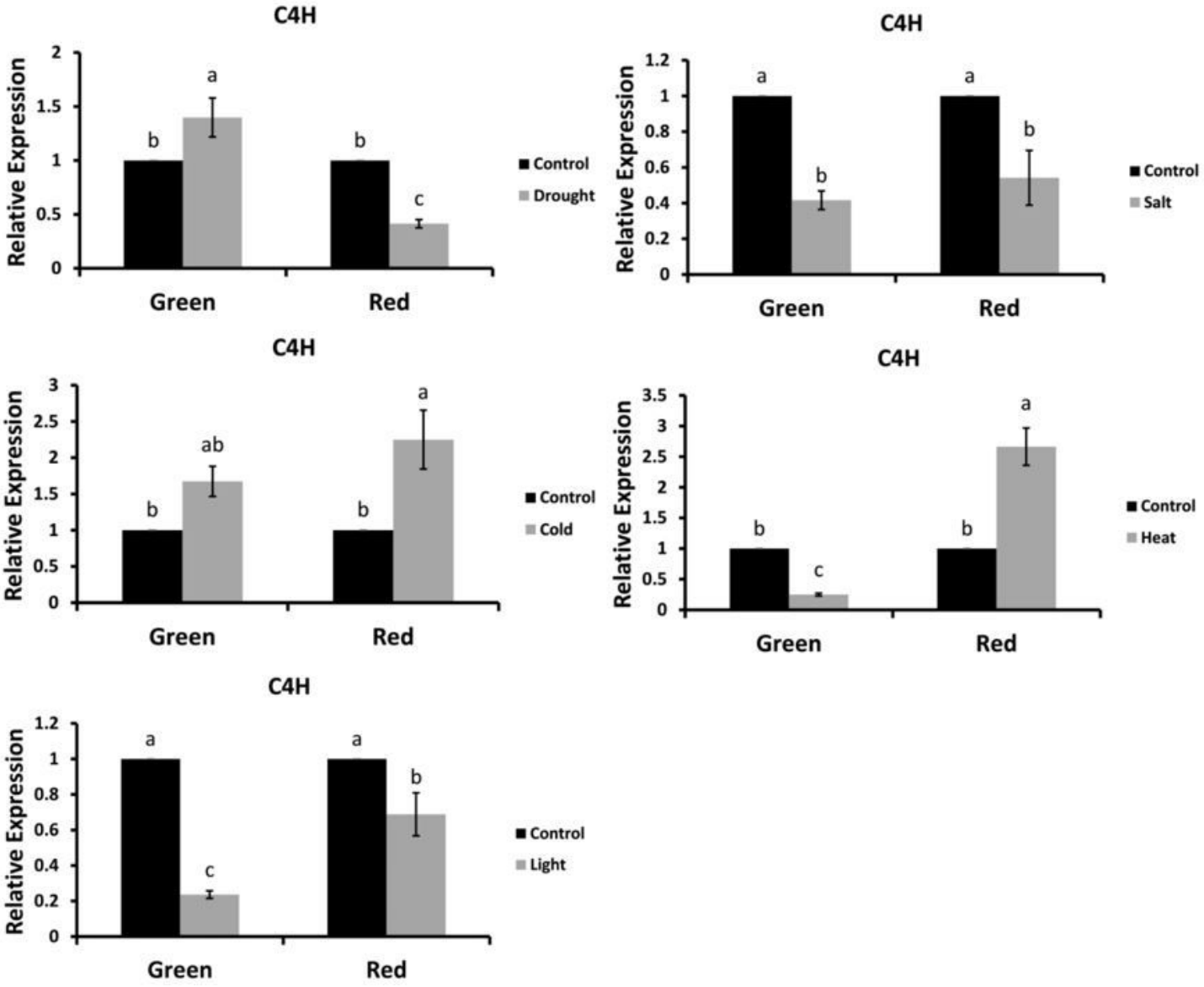

Figure 6

Expression pattern of C4H gene in red and green cultivars of basil under different abiotic stresses. Bars show standard errors. Letters above the error show significant differences. Means with the same letter are not significantly different. The Tukey's test was used for multiple comparisons of means at $P < 0.05$.

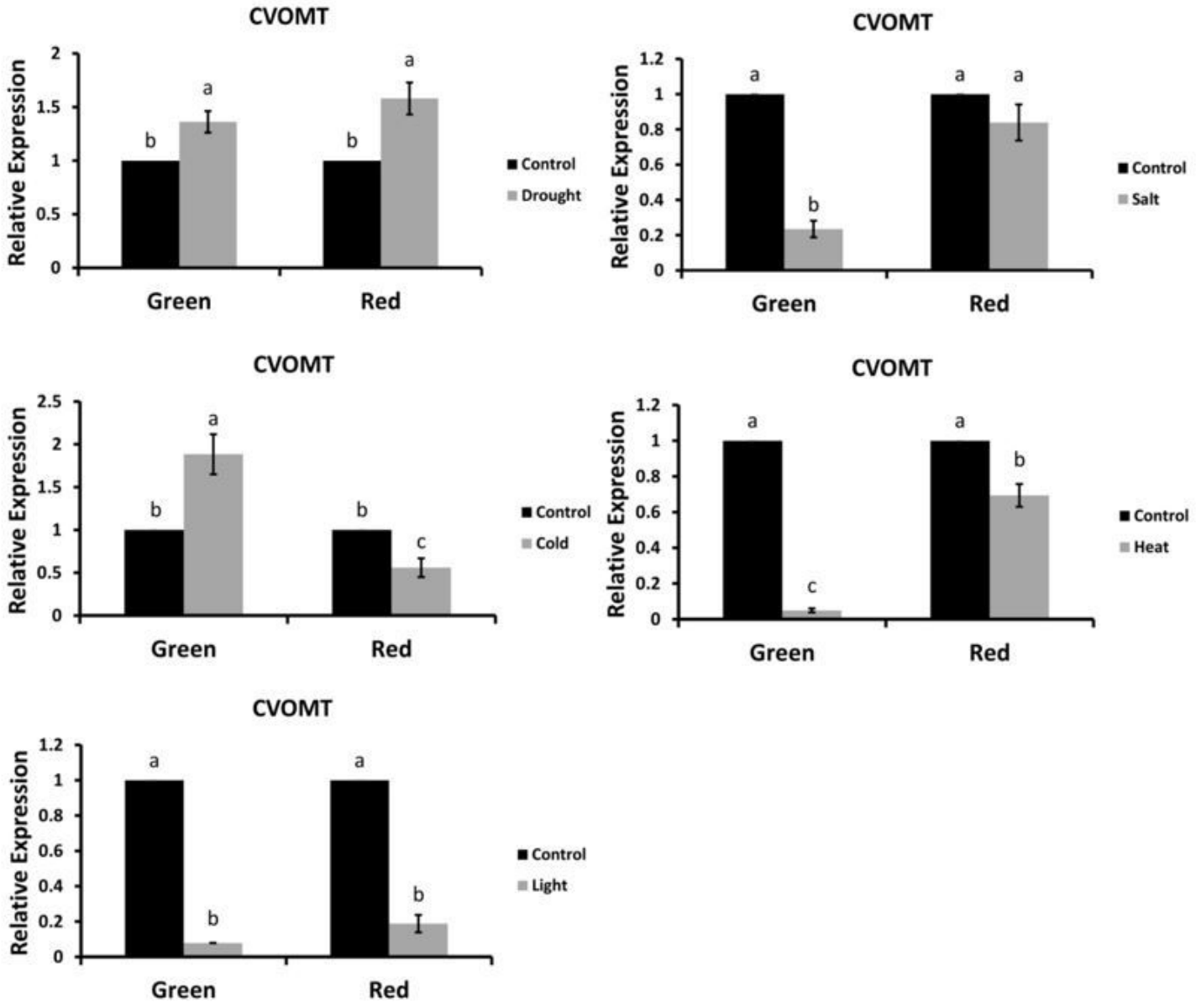

Figure 7

Expression pattern of CVOMT gene in red and green cultivars of basil under different abiotic stresses. Bars show standard errors. Letters above the error show significant differences. Means with the same letter are not significantly different. The Tukey's test was used for multiple comparisons of means at $P < 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Keygenesinthephenylpropanoids....blin.rar
- Supplementary.docx