Identification and functional characterization of the CVOMTs and EOMTs genes promoters from Ocimum basilicum L.

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Abstract
Methyl chavicol and methyl eugenol are important phenylpropanoid compounds previously purified from basil. These compounds are significantly enhanced by the water deficit stress-dependent mechanism. Here, for the first time, pObCVOMT and pObEOMT promoters were extracted by the genome walking method. They were then cloned into the upstream of the β-glucuronidase (GUS) reporter gene to identify the pattern of GUS water deficit stress-specific expression. Histochemical GUS assays showed in transgenic tobacco lines bearing the GUS gene driven by pObCVOMT and pObEOMT promoters, GUS was strongly expressed under water deficit stress. qRT-PCR analysis of pObCVOMT and pObEOMT transgenic plants confirmed the histochemical assays, indicating that the GUS expression is also significantly induced and up-regulated by increasing density of water deficit stress. This shows these promoters can drive inducible expression. The cis-acting elements analysis showed that the pObCVOMT and pObEOMT promoters contained dehydration or water deficit-related transcriptional control elements.

Key message
Activity of the full-length promoters of ObCVOMT and ObEOMT exhibited that the isolated region of these promoters can induce the GUS expression in the leaves of transgenic tobacco under water stress levels.

Keywords Ocimum basilicum · pObCVOMT · pObEOMT · Water deficit stress · Cis-regulatory elements

Abbreviations
CVOMT  Chavicol O-methyltransferase
EOMT  Eugenol O-methyltransferase
FC  Field capacity
GUS  β-Glucuronidase
PLACE  Plant cis-acting regulatory DNA elements
qRT-PCR  Quantitative real-time PCR
TF  Transcription factors
5′-UTR  5′-Untranslated region
X-gluc  5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Introduction
One class of essential compounds, volatile phenylpropenes (methyl chavicol and methyl eugenol), make up the essential oils in aromatic, and herbaceous plants. Methyl chavicol and methyl eugenol as flavor and fragrance compounds have important roles in plants, including as insect pollinator attractants or repellent, and possess antibacterial and fungicidal properties (Devi et al. 2010). Volatile phenylpropanes are the major constituents of Ocimum basilicum (one genus from the Lamiaceae family regarded as the most important medicinal and ornamental plant for the production of
essential oils), which have a distinctive aroma (Gang et al. 2001).

Chavicol and eugenol are the principal bioactive components of the defensive arsenal of the Ocimum species, which act as signal molecules among plants, humans, and microbes. These compounds are widely used in the food industry as food additives, in fragrance, and medical sectors (Simon et al. 1990; Nishida 2014). These plant-based metabolites serve as antibacterial compounds against effective antifungal agents, significant food-borne pathogenic bacteria, and good nematicides at low concentrations (Devi et al. 2010; Šimović et al. 2014).

The biosynthetic pathways of these phenylpropenes and their derivatives have not yet been identified in detail. Briefly, the primary steps begin with the production of 4-coumaric acid, cinnamic acid, ferulic acid and caffeic acid in the general phenylpropanoid pathway (Dixon et al. 2002; Ehlting et al. 2006). The following stages are followed by the subsequent biosynthetic pathways through deamination, methylation, decarboxylation stages, and adding methoxyl and hydroxyl functional groups (Dixon et al. 2002; Gang et al. 2001). The final stage in the biosynthesis of phenylpropene, methyl chavicol and methyl eugenol is catalysis by eugenol O-methyltransferase (EOMT), and chavicol O-methyltransferase (CVOMT) enzymes (Vogt 2010).

Owning these essential roles in plant defense systems and their applications in different industries, biosynthesis of these two phenylpropenes has been well understood at the molecular and biochemical levels in O. basilicum (Gang et al. 2001). Renu et al. (2014) showed the high expression of gene encoding EOMT in developing tissues, indicating a high level of methyl eugenol synthesis (Renu et al. 2014). In our previous studies, we identified basil water stress-responsive genes (i.e., CVOMT and EOMT), and we characterized their expression patterns in the three basil cultivars during water deficit stress treatments (Khadkan et al. 2017). Many studies describing the biosynthesis of methyl chavicol and methyl eugenol constituents, with the expression of CVOMT and EOMT genes as key genes involved in the pathway, the role of origin of plants, and drought stress having been studied (Pirbalouti et al. 2013; Al-Kateb and Mottram 2014; Khadkan et al. 2016, 2017).

Similarly, the biosynthesis of volatile phenylpropenes e.g., methyl chavicol and methyl eugenol occur at different growth stages of the Ocimum species, and are regulated under abiotic stresses (ref). Hence, the identification of specific gene promoters owning tissue-specific and stress-responsive elements to control specific expression of transgenes in new genetically-modified crops without facing the adverse effects associated with the CaMV 35S promoter is highly desirable (Tu et al. 2009).

Understanding the transcriptional regulation of the gene encoding rate-limiting enzymes of a specific secondary metabolite pathway could be achieved by controlling the overexpression of particular trans-factors of the crucial enzyme genes playing a notable role in regulating such enzymes under certain environmental conditions (Butelli et al. 2008). Therefore, characterizing the promoter structure and mapping the functional sequence domains of a gene can be employed to provide a synthetic or modified promoter. These promoters remain active under specific environmental conditions or positively respond to the action of particular trans-factors in the production of unique metabolites with significant medical importance. As a result, by using such promoters, greater concentrations of a particular secondary metabolite can be achieved (Dey et al. 2015).

The effect of transcriptional regulation on response to biotic and abiotic cues and reprogramming the metabolic processes in plant development has been noticed in numerous plant systems (Spitzer-Rimon et al. 2012). However, our little knowledge about the regulatory networks controlling the secondary metabolism comes mainly from studies on the transcriptional regulators participated in the biosynthesis of alkaloids in response to glycosylates (Fits and Memlink 2000; Burow et al. 2010; Sønderby et al. 2010), flavonoids (Feller et al. 2011), benzenoids (Verdonk et al. 2005) and anthocyanin (Shaipulah et al. 2016).

Despite tremendous efforts devoted to study the volatile phenylpropanoid biosynthesis pathway, only a few TFs which regulate the pathway structural genes expression have yet been determined. In Petunia hybrid, as a model system for studying anthocyanin and volatile phenylpropanoid biosynthesis, the functions of R2R3-MYB TFs and ODORAN1 (ODO1) proved to be able to regulate the levels of the genes encoding volatile and nonvolatile phenylpropanoid biosynthesis pathways (Verdonk et al. 2005; Spitzer-Rimon et al. 2012; Klahre et al. 2011). Interestingly, RNA interference (RNAi) results revealed the key role of ODO1 in the regulation of the shikimate pathway towards benzenoid production (Verdonk et al. 2005).

Although methyl chavicol production in glandular trichomes (on the aerial parts’ surface) has been studied, regulation of the volatile phenylpropanoid structural pathway engaged in production of eugenol in O. basilicum has not been clarified. For the first time, we isolated and characterized CVOMT and EOMT genes promoters and their 5′-untranslated region (5′-UTR) from O. basilicum in silico. We also investigated their functions by examining the stable transformation of tobacco (Nicotiana tabacum var. Samsun) with a promoter-GUS fusion construct. Finally, the specific expression patterns of the promoters in the leaves of transgenic tobacco plants were studied under water deficit stress using histological analyses.
Materials and methods

Plant materials

Basil seeds, obtained locally (originating from Jahrom City, Iran), were grown in the research greenhouse at a temperature range of 20–30 °C under natural lighting. Genomic DNA was extracted from basil leaves (approximately 2-week-old seedlings) using the CTAB method (Xiao et al. 2002). First, DNA concentration and purity were specified by a NanoDrop® ND-1000 spectrophotometer. Next, the samples with a 260:280 ratio ranged 1.9 to 2.1 were selected and used in the analyses. Tobacco (N. tabacum var. Samsun) seedling leaves (1-month-old) grown on sterile MS medium under standard growth chamber conditions were applied in leaf-disc transformation using the Agrobacterium tumefaciens-mediated method.

Promoter cloning and sequence analysis

The pObCVOMT and pObEOMT promoters were extracted from the basil genomic DNA using the genome walking method. For this purpose, the genomic DNA with a restriction enzyme was cleaved to generate a blunt end followed by a complementary adaptor ligation for adaptor-dependent extension (Clontech, USA). When the basil genomic DNA was thoroughly digested by two restriction enzymes (DraI and EcoRI), it was connected to the adaptors with T4 ligase to construct genomic libraries. The two stranded-ligated adaptors having a blunt terminus were formed by annealing an equal amount of adaptors (AP1; Adaptor-1 and Adaptor-DraI and AP2: Adaptor-1 and Adaptor-EcoRI) (Table 1). The digested DNA genomic of basil was ligated separately to the adaptors (AP1 and AP2). Then the adaptor-linked genomic DNA was applied as a preset model for amplification of PCR using gene-specific primer 1 (GSP1-C, GSP1-E for ObCVOMT and ObEOMT, respectively), designated based on the 5' end of the coding region of the reference sequence (GenBank Accession Nos. AB530137 and AF435008 for ObCVOMT and ObEOMT, respectively) and adaptor–primer.

Common PCR was performed using a Bio-Rad thermocycler in a final volume of 20 μL, which contained 0.2 mM of dNTPs, 1 x PCR buffer, 1 unit of Taq DNA polymerase (5 U/μL), 0.4 μM of each primer, and 200 ng of cDNA. The reaction was boosted by a touch-down PCR protocol with the temperature profile of 5 min at 94 °C and followed by 5 cycles of 45 s at 94 °C, 90 s at 72 °C, and 45 s at 63 °C (reducing 1 °C per cycle). Then it was subjected to 30 cycles of 45 s at 60 °C, 45 s at 94 °C, and 90 s at 72 °C and an ultimate extension of 10 min at 72 °C. A 20-fold diluted PCR product was applied as a preset model to boost the second PCR using the nested primer (GSP2-C and GSP2-E for ObCVOMT and ObEOMT, respectively) and adaptor–primer. Except for the nested primer, the components of the second PCR amplification reaction and reaction temperature conditions were the same as those of the first amplification. 1% agarose gel electrophoresis was used to analyze the PCR products, and UV fluorescence after staining with ethidium bromide was employed to visualize them. Accordingly, 616-bp and 1028-bp amplicons of pObCVOMT and pObEOMT were purified using the glass milk method (Sambrook and Russell, 2001) ligated to a pTG19-T PCR cloning vector (Vivantis, Korea), and chemically transformed in competent DH5α cells (Novagen, USA). Finally, the extracted plasmid was used to determine the sequence [Bioneer Biotechnology Co. Ltd. (Korea)].

Table 1  Sequences of adaptors and primers used in the present study

| Adaptor/primer name          | Adaptor/primer sequences                  | Modification          |
|------------------------------|-------------------------------------------|-----------------------|
| Adapt-1                      | 5’-ACTATAGTGACTGCTGTGAGGCCGCTGCTGTT-3’    | –                     |
| Adapt-DraI                   | 5’-AACCGACTGCC-3’                         | 5’-Phosphate and 3’H2N|
| Adapt-EcoRI                  | 5’-AATTACCAGCCC-3’                        | 5’-phosphate and 3’H2N|
| GSP1-C                       | 5’-TAT CGG GTA TGC CTA ATT GAA TTG CAC AT-3’ | –                     |
| GSP2-C                       | 5’-GAA GTT GCT CAG TCG ACA ATG AAA TAT CCA T-3’ | –                     |
| GSP1-E                       | 5’-ATGGGATGGATTGAGTAATTTGGGG-3’           | –                     |
| GSP2-E                       | 5’-TAAGGACATTTGATTTGCAAGGCGTAC-3’         | –                     |
| Adaptor–primer               | 5’-ACTGCTGTGCTGAGGCC-3’                   | –                     |
| pObCVOMT-HindIII-F           | 5’-CCC AAG CTT AAA TGG GTC AAT TTT GGG TTG-3’ | –                     |
| pObCVOMT-BamHI-R             | 5’-CCG GGA TCC TGG ATG TAA TTT ATG TGG TAA ACTAGG-3’ | –                     |
| pObEOMT-indIII-F             | 5’-CCC AAG CTTGAA ATT GTC GCT GGA GAG-3’   | –                     |
| pObEOMT-BamHI-R              | 5’-CCG GGA TCC GCT GGG CTT GGT GAT GTC A-3’ | –                     |
| uidA-F                       | 5’-TGATAGCGCGTGACAAAAA-3’                 | –                     |
| uidA-R                       | 5’-CGAAATATTTTCCCCTTGCAC-3’               | –                     |
The sequence data was aligned by the BLAST algorithm option available at NCBI (http://www.ncbi.nlm.gov/). The putative transcription start site (TSS), cis-acting regulatory elements and their positions were predicted by the PLACE software (Higo et al. 1999), and the Signal Scan Program PlantCARE database (Lescot et al. 2002).

Construction of the pObCVOMT::GUS expression vector

The isolated pObCVOMT and pObEOMT were amplified with primers pObCVOMT-HindIII-F, pObCVOMT-BamHI-R and pObEOMT-HindIII-F, pObEOMT-BamHI-R, employed as preset models (Table 1). These promoters contained HindIII and BamHI-R restriction sites, respectively (underlined). The obtained amplicon (with a double restriction site) was ligated to the sequencing vector of pTG19 and digested by good places. The digested yields were fused to the GUS reporter gene, where pTG19-pObCVOMT, pTG19-pObEOMT, and pBI121 binary vector as the mainframe was digested with and BamHI and HindIII. Subsequently, the pObCVOMT and pObEOMT fragments were connected to digested binary vector pBI121 to substitute the CaMV 35S promoter individually. The obtained pObCVOMT-and-pObEOMT promoter fragments were individually cloned to the upstream of the GUS reporter gene to examine the GUS gene expression under water deficit stress (Fig. 1). Sequencing verified all the constructs.

Tobacco transformation and growth condition

Two plant expression vectors for each promoter, pObCVOMT::GUS, pObEOMT::GUS, and pBI121 (p35S::GUS), were introduced into A. tumefaciens strain C58 by the freeze–thaw method (Sambrook and Russell, 2001). These vectors were subsequently moved into A. tumefaciens (strain C58) according to the method described by Wang (2006). Briefly, A. tumefaciens from an individual colony was grown at 28 °C for 48 h in 25 mL of YEP medium (5 g/L Bacto peptone, 6 g/L yeast extract, 2 mM MgSO₄ and 5 and g/L sucrose) supplemented with kanamycin (50 μg/mL) and rifampicin (50 μg/mL) at 28 °C. It was then added to 50 mL of induction medium (YEP medium and 20 mM acetosyringone) with similar antibiotics and grown again. After adjusting to an OD600 of approximately 0.7–1, on the following day, 55 g/L and 20 μM were added into the bacterial cells medium and incubated with gentle agitation (20 rpm) at room temperature for nearly 1 h. Agrobacterium cells were transferred into the approximate 0.5 cm² 6-week-old tobacco leaf discs using the agroinfiltration method in 250 mbar vacuum condition for 20 min (Wang 2006) and rapidly eliminated the liquid and bacteria. After co-cultivation at 28 °C for 48 h in dark, the infected discs were moved to the selection medium [MS salt; BA (1 mg/L), kanamycin (100 mg/L), NAA (0.1 mg/L), sucrose (30 g/L), agar (8 g/L), and cefotaxime (150 mg/L)]. The transformants were placed in a 1/2 strength MS rooting medium containing kanamycin (100 mg/mL). Positive transgenic plants were screened on the selective medium, and amplification of PCR was applied to confirm the positive plants. The respective transformed plants (pObCVOMT::GUS, pObEOMT::GUS, and p35S::GUS) were grown in a pot having sterile vermiculite and perlite (2:1) in a tissue culture chamber at controlled temperature (22 °C ± 2) under an 8-h dark/16-h light cycle. Ultimately, three transgenic plants for each construct were used for water deficit stress.

Water deficit stress treatments

Before water deficit stress treatment, seedlings of 2 months old were transplanted in new plastic pots having sandy-loam soil and allowed to adapt to the soil circumstances and further grow under optimum conditions for approximately two weeks until 25 days old. Considering the water deficit stress treatment, the three stress levels were set based on field capacity (FC) through withholding water and labeled

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**Fig. 1** Schematic representation construction of the pObCVOMT::GUS and pObEOMT::GUS vectors. The pTG19-pObCVOMT, pTG19-pObEOMT and pBI121 binary vector were digested with HindIII and BamHI. In the final transformation vectors, the GUS gene is placed under the control of pObCVOMT and pObEOMT promoters. **LB** left border, **NPTII** neomycin phosphotransferase II, **GUS** (β-glucuronidase) reporter gene, **NOS** nopaline synthase terminator, **RB** right border
as W1: 75% FC, W2: 50% FC and W3: 25% FC with three replicates. Plant sampling was done after water deficit stress treatment for 1 week. For control treatment, the plants were developed with no water deficit stress at the time, similar to water deficit stress.

**Histochemical GUS assay**

Transgenic tobacco leaf tissues were histochemically studied for GUS activity according to the staining process reported by Jefferson et al. (1987) with some modifications. The samples were submerged in X-Gluc buffer [100 mM phosphate buffer (pH 7.0) and 1 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (X-Gluc), 10 mM EDTA, 0.5 mM potassium ferrocyanide, and 0.006% (v/v) Triton-X 100] for 3–24 h at 37 °C. Following the staining, the tissues were extensively rinsed in 70% ethanol to remove chlorophyll and finally photographed using a digital camera under a stereomicroscope (Leica MZFL III).

**Transcription level analysis using quantitative real-time PCR (qRT-PCR)**

To validate the results obtained with histochemical GUS assay and the analysis the activity of the pObCVOMT and pObEOMT, qRT-PCR assays were carried out using the three water deficit levels-treated RNA samples of the resulted three transgenic plants expression constructs (pObCVOMT::GUS, pObEOMT::GUS, and p35S::GUS).

The leaf total RNA samples of the three treated transgenic plants (a total of six plants for each treatment) and the control group were isolated using a pBIOZOL reagent (Invitrogen), as indicated by the manufacturer’s instructions. The total RNA concentration was determined spectrophotometrically using a NanoDrop® ND-1000, while the RNA integrity of each sample was examined by an agarose gel electrophoresis-based system. The cDNA synthesis reaction was initially performed using the Revert-Aid™ First Strand cDNA Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany) following the manufacturer’s recommendations. The gene-specific primers sequences of the uidA (GUS) were designed based on the corresponding gene using Primer3 software (http://frodo.wi.mit.edu/primer3/) (Table 1). Next, qRT-PCR amplification assay was carried out using a QIAGEN’s real-time PCR system (Rotor-Gene Q), in a 20 µL final volume consisted of 1.5 µL of diluted cDNA, 8 µL EvaGreen Master Mix (containing EvaGreen Dye, Solis BioDyn, Germany), 0.25 µM of each primer (forward and reverse) followed by adding PCR-grade water. The qRT-PCR reaction mixtures were done with a temperature profile of 15 min at 95 °C, 45 cycles of 15 s at 96 °C, 20 s at the specific annealing temperature for each primer, and 20 s at 72 °C. Additionally, as a housekeeping (reference) gene, the Actin gene (GenBank Accession No. AB002819) was also used (Table 1) (Renu et al. 2014). For every reaction, the efficiency of PCR was calculated using QIAGEN’s real-time PCR machine (Rotor-Gene Q), and the values equal to or greater than 0.8 (80%) were applied for extensive analyses. The experiments were performed in triplicate, and statistical analysis of gene expression was conducted by REST software (http://rest.gene-quantification.info).

The data represent the mean of the three replicated treatments were subjected to analysis using analysis of variance (ANOVA) to assess the significant differences between treatments using Duncan’s multiple range tests ($p \leq 0.05$).

**Results**

**Cloning and sequence analysis of the CVOMT gene promoter (pObCVOMT)**

A 616-bp upstream fragment of the ObCVOMT gene (Fig. 2A) was isolated from O. basilicum genome by genome walking technology based on PCR and nested specific primers from ends of the identified region randomly anneal with the genome unknown flanking fragments (Liu and Chen 2007). Blast results of the obtained 5′ upstream sequence and 5′-UTR region of ObCVOMT showed the overlapping sequences with maximum identity. It was named as pObCVOMT and registered in GenBank (Accession No. KT310085). Sequence analysis of ObCVOMT showed the transcriptional start site at − 39 bp upstream of the translation initiation codon (ATG), according to both consensus sequence for TSS in eukaryotes and the proposed distance between TATA-box and TSS (32 ± 7) (Joshi 1987). Therefore, adenine was designated as TSS (Fig. 3). The predicted results of promoter elements in ObCVOMT demonstrated the putative plant cis-acting regulatory elements and their functions (Table 2). The results showed that TATA-box (TATATAT) was localized − 34 nt from the putative TSS, which corresponded to the distance of TATA-box from the transcription initiation site and characteristics of the plant promoters (Joshi 1987). Another highly conserved eukaryotic transcriptional cis-element in core promoter regions (CAAT box) was located at − 79 to − 83 bp (CCCAAT) in the ObCVOMT gene promoter region. The genomic DNA upstream region of the TSS was examined to identify biotic and abiotic stress-related cis-elements in the ObCVOMT promoter region (Table 2; Fig. 3). Eleven types of putative cis-acting elements were detected in the promoter region and were more consistent with the ones previously reported in the activation of elicitor-responsive and defense-related genes in plants (Behnam et al. 2013; Xu et al. 2015; Dass et al. 2016; Wang et al. 2016a, b; Conforte et al. 2017; Xue et al. 2018; Wang et al. 2019). For example, one dark-induced
and dehydration stress senescence (ACGTATERD1) (Simpson et al. 2003), a drought-responsive element (CBFHV) (Svensson et al. 2006), and dehydration, high salinity, cold and drought-responsive cis-elements were found upstream of the promoter (Xie et al. 2005; Rushton et al. 2010). Many cis-active motifs with response to dark and light, including four GT1 consensus motifs, were together with respective trans-acting elements and GT1, which were recognized and participated in several regulatory processes (Zhou 1999; Civan and Svec 2009). However, the other critical regulatory elements related to the response to light, such as Pc-CMA2c and E-box (Hartmann et al. 2005), were also identified, without the corresponding trans-factors. Moreover, the cis-acting elements related to hormone responsiveness were detected (Fig. 3). The CGTCA motif, LTRECOREATCOR15, overlapping with GT1-Box and DRE-box, proved to be a MeJA, SA-responsive element (TCA-element), and abscisic acid-responsive element (ABRE) (Lopez-Molina and Chua 2000;
Mena et al. 2002; Ross et al. 2004; Nakashima et al. 2006; Kaplan et al. 2006), respectively, in the promoter region of ObCVOMT.

The ObCVOMT promoter contains one conservative binding site (MBS motif) identical to the plant MYB transcriptional factors, which included in the drought-induced and flavonoid biosynthesis gene expression. Moreover, four Dof TF binding site was observed in the upstream of the ObCVOMT gene, which may function as a regulatory factor of the gene in the signal response of auxin, and jasmonic acid or ethylene (Baumann et al. 1999; Yanagisawa and Schmidt 1999; Liao et al. 2015) in other plant promoters, which is also thought to promote strong transcriptional activity. In addition, we found MYCCONSENSUSAT (MYC-core) overlapped with

| Site name                  | Sequence          | Number of cis-elements (+) strand | Distance from TSS | Function                                                                 |
|----------------------------|-------------------|-----------------------------------|-------------------|--------------------------------------------------------------------------|
| TATA-box                   | TATATATA          | 1                                 | −35               | Common cis-acting element in enhancer regions                             |
| CAAT-box                   | CCAAT             | 1                                 | −79               | Core promoter element for transcription start                             |
| CGTCA                      | CGTCA             | 3                                 | −134, −156, −296  | Cis-acting regulatory element involved in the MeJA-responsiveness         |
| MBS                        | GTCAAC            | 1                                 | −393              | MYB binding site involved in drought-inducibility and flavonoid biosynthesis |
| Pc-CMA2c                   | GCCCACGCA         | 1                                 | −171              | Part of a light responsive element                                        |
| GATA-motif                 | GATA              | 0                                 | −324              | Part of a light- and tissue specific-responsive element                    |
| EBOXBNNAPA (E-box)         | CANNTG            | 1                                 | −356              | Cis-acting elements recognized by R2R3-MYB, BZIP, and BHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes |
| MYCCONSENSUSAT (MYC-core)  | CANNTG            | 1                                 | −353              | MYC recognition site involved in dehydration and cold responsiveness      |
| CBFHV                      | GTCGAC            | 0                                 | −353              | A dehydration-responsive element (DRE)                                    |
| LTRECOREATCOR15            | CCGAC             | 1                                 | −368              | A cold, drought and ABA-responsive element                               |
| DRE                        | ACCGAC            | 1                                 | −369              | Dehydration, high salinity and cold responsive element                     |
| ACGTATERD1                 | ACGT              | 1                                 | −187              | Involved in upregulation by dehydration stress and dark-induced senescence|
| W-box                      | TGACT             | 0                                 | −389              | WRKY binding site, involved in many physiological processes               |
| GT1-box                    | GRWAAT            | 1                                 | −49, −79, −326    | Consensus GT-1 binding site in many light-regulated genes and influence the level of SA-inducible gene expression |
| DOF-box                    | AAAG              | 1                                 | −414, −448, −469, −511 | Auxin-responsive element                                                  |
| WRKY-binding site          | TGAC              | 1                                 | 133, 155, 175, 305, 399 | WRKY binding site, involved in many physiological processes               |
E-box and W-box at −353, −389 and −356, respectively, in the ObCVOMT promoter (Fig. 3) as the conservative binding motif of MYC, bHLH (Abe et al. 1997, 2003; Wang et al. 2016a, b), WRKY (Xie et al. 2005; Rushton et al. 2010) TFs, respectively. The presence of consensus E-box and W-box binding site in the ObCVOMT promoter may reflect that basil CVOMT is subjected to autoregulation or recognized, and modulated to R2R3-MYB, BZIP, BHLH and WRKY protein members. Previous studies showed, these TFs participate in the transcriptional reprogramming associated with resistance to various stresses, tissue-specific regulation of phenylpropanoid biosynthesis genes and many physiological processes (Eulgem et al. 2000; Rushton et al. 2010), suggesting that the transcription rate of O. basilicum CVOMT may potentially depend on exposure to drought stress, hormone, dark and light (Table 2). The outcome of the in silico analysis indicated that the ObCVOMT promoter activity could be controlled through the numerous external and internal plant environments, which is suggestive of an essential role in the activation of the biological defense process.

Cloning and analysis of the 5′ flanking region of ObEOMT

With the help of nested gene-specific primers for sequence of the gene as close to the 5′ end as possible (the first exon of the gene), we amplified the 5′ flanking fragment (1208 bp) of the EOMT gene of O. basilicum using a PCR-based method, as described in Sect. 2. It was designated as pObEOMT and its accession number at GenBank is KY492343. The TSS of pObEOMT was anticipated to be 157 bp upstream from the start codon ATG and was defined as “+1”. An approximately 1208 bp-long PCR product was sequenced and submitted to the PLACE and PlantCARE database search programs to anticipate putative cis-elements in the promoter region of pObEOMT in the gene expression regulation. Function and location of the anticipated cis- elements are shown in Table 3 and Fig. 3. Core promoter consensus sequences CAAT box and TATA box are the well-known motifs in the 5′ flanking region of eukaryotic genes. Multiple core cis-acting elements, including four sequences analogous to TATA box and nine enhancer cis-acting elements CAAT-box, were identified at numerous positions from the TSS in pObEOMT; and all of them are marked in Fig. 4.

The most potential putative TATA box was located at −27 (ATATAA), similar to the most of other eukaryotic genes (Zou et al. 2011). Moreover, a series of potential regulatory elements which simplify the inducible expression of the ObEOMT were identified. Four types of cis-acting elements related to light responsiveness were observed in ObEOMT 5′-flanking region: one site of AE-box, one site of ATCT-motif and two sites of GT1-motif. Besides, there were several regulatory elements for stress defense and hormone responsiveness, including five putative types of MYC-binding sequences (MYC-core) in transcriptional control in abscisic acid- and drought-response, MYB binding site in drought inducibility gene expression, eight types of hormone-responsive elements [ABRE (abscisic acid responsive element), TCA-element (salicylic acid responsive element) and PYRIMIDINEBOXOSRAMY1A (response to gibberellin)], CGTCA-motif and TGACG-motif (response to MeJA), an factor related to low temperature-responsive (LTRE-motif) and WRKY binding site in numerous physiological processes (Zhou 1999; Rushton et al. 2010; Lopez-Molina and Chua 2000; Abe et al. 2003; Kaplan et al. 2006; Agarwal et al. 2006; Liao et al. 2015; Wang et al. 2019).

Tobacco transformation and screening transgenic

To understand the functional roles of the pObCVOMT and pObEOMT promoters and theirs response to drought stress, the expression of GUS fusion reporter was assessed to verify the transcriptional activity of the promoters (Fig. 1). For a better comparison of expression patterns under water deficit stress, p35S::GUS transgenic tobacco (including pBI121 with 35S promoter construct) and non-transgenic plants were used as positive and negative controls, respectively. The three resulting plant expression constructs, the pObCVOMT::GUS, and pObEOMT::GUS, were utilized for Agrobacterium-mediated transformation and regeneration of fully expanded leaves of tobacco plants for promoter functional analysis. Six independent kanamycin-resistant transgenic plants were analyzed using PCR, the positive plants from each group were selected for further investigation and grown in the greenhouse.

Water-stress induced expression of the ObCVOMT and pObEOMT promoter-GUS reporter in tobacco leaf tissues

After selection, the activities of pObCVOMT and pObEOMT promoters underwent stable expression assessment using the Agrobacterium-mediated leaf-disc transformation method in different plant tissues. All the positive transgenic plants were evaluated for pObCVOMT-driven GUS by histochemical GUS staining, and the GUS activities at transcription level after water deficit stress treatment (25, 50 and 75% FC) (Fig. 5A, B). Negative control tobacco leaves showed no GUS activity. As shown in Fig. 5, the 35S::GUS transgenic plants submitted to all of the three water deficit stress levels showed intense GUS activity. Regarding transformant bearing of the pObCVOMT::GUS construct, the maximum blue staining of the leaf was shown under 25% FC (W1), while for W1 and W2, some blue spots were achieved (Fig. 5B). Histochemical staining of GUS activity approved that the GUS expression driven by pObCVOMT at W3 was higher
than pObCVOMT::GUS expression under W1 and W2 treatment. In other words, the water deficit stress treatment of pObCVOMT::GUS transgenic plants led to variable expression patterns at different levels.

Relative expression analysis of pObCVOMT::GUS transgenic plants was examined by qRT-PCR analysis. The normalization of the level of uidA (GUS) expression to remain stable condition under various levels of water deficit stress
was set by the actin gene. As Fig. 5C is shown, for transformant bearing of the *pObCVOMT**:GUS construct, relative expression ratio of GUS increased from the inception of water deficit stress (W1; 1.35-fold), and gradually increased for W2 (2.70-fold) and lastly boasted to its maximum level for W3 (~27.41-fold). This suggests that GUS gene was expressed in the transgenic plant leaves, and the role of the drought-responsive elements can validate the regulation of *pObCVOMT* (gene expression data) under water deficit stress conditions (Fig. 5C).

The results from GUS histochemical assay revealed that when transgenic tobacco was exposed to high levels of water deficit stress (W3), the highest level of GUS activity as very intense staining was observed. However, the obvious levels of GUS activity was detected in the transgenic plants under W1 and W2 water deficit stress levels (Fig. 5B). The results showed, *pObEOMT* was unable to drive the GUS gene expression with different levels under W3 level. qRT-PCR analysis of *pObEOMT* transgenic plants confirmed the histochemical assays, indicating that the *uidA* expression is also significantly induced and up-regulated (p < 0.05) by increasing the density of water deficit stress (Fig. 5B). We infer that the presence of the maximum number of MYC-recognition site as the main dehydration-responsive elements also play an essential role in the response of water deficit stress and may elevate high levels of *uidA* expression under W3 (5.12-fold). As expected, the activity of GUS in positive control tobacco was transformed with *Agrobacterium*-harboring *p35S::GUS* construct, showing robust activity, in contrast, no GUS activity was found in non-transgenic tobacco (negative control) (Fig. 5B, C).

**Discussion**

Promoters are essential elements that regulate gene expression both temporally and spatially and induce transcription (Juven-Gershon and Kadonaga 2010). Most studies on gene function and transgenic breeding have been carried out with focused promoters and distinct activity patterns. Among the available strategies, the spatial- or tissue-specific promoters need to be employed in transgenic research to reach an efficient expression of transgenes encoding the rate-limiting enzymes of a particular secondary metabolite biosynthesis pathway in homologous and heterologous hosts in particular plant tissues or at specific times (Zhou et al. 2012). Therefore, the applying of particular promoters (tissue, developmental stage-specific, and stress-responsive) is expected to achieve efficient expression of promising metabolites and increase the detection of suitable plants in a concerted way. The environmentally inducible gene expression of transgenes is transcriptionally regulated by the frequency of cis-acting regulatory elements to bind TFs, which may play a critical role in driving the more significant accumulation of a particular secondary metabolite (Dey et al. 2015).

We have previously shown the levels of mRNAs encoding *CVOMT* and *EOMT* genes are increased concurrently with methyl chavicol and methyl eugenol compounds as volatile phenylpropanes, respectively. The strong positive correlation between the expression level of these genes and the final compounds biosynthesized during the water deficit stress indicates a superficial relationship (Khakdan et al. 2017).

To understand the mechanism through which the *ObCVOMT* and *ObEOMT* genes expression is regulated when basil is subjected to water deficit stress, we initially isolated 616-bp and 1208-bp long promoters of *ObCVOMT* and *ObEOMT*, respectively (Fig. 2) and determined numerous cis-regulatory elements in them. These elements are critical molecular switches in the *ObCVOMT* and *ObEOMT* genes’ regulatory networks, according to the PLACE and PlantCARE databases (Figs. 3, 4; Tables 2, 3). Consequently, we functionally analyzed the *ObCVOMT* and *ObEOMT* promoters to determine the role of the predicted central regions and the activity of the full-length isolated promoter influencing GUS gene expression during the response to water deficit stress through an *Agrobacterium*-mediated stable evaluation in tobacco leaves.

The isolated 5'-flanking sequence of the *ObCVOMT* and *ObEOMT* genes comprise a putative promoter sequence of ~571 and ~1178 bp upstream of the translational initiation site (ATG), respectively, and TATA-box and CAAT-box are near the putative TSS. A core promoter was considered to be the DNA region that is essential to guarantee the accurate start of the transcription process by RNA polymerase, which is often presumed to be a special region with wide diversity in structure and function (Javen-Gershon and Kadonaga 2010). TATA-box was predicted at ~35 bp upstream of the TSS in the promoter of *ObCVOMT*, while three TATA-boxes (TATA, ~27 to ~30 and ~523 to 526 bp; ATATAA, ~99 to ~104 bp; TAAAGATT, 410 to 417 bp) based on sequence homologies are identified in the *pObEOMT*, which may be vital for promoter activity. Another interesting characteristic in *pObCVOMT* and *pObEOMT* promoters is their other conserved cis-acting elements, including CAAT-box, at similar arrangement (Tables 2, 3). As can be seen in Tables 2 and 3, this box is located between ~79 and ~83 and nine positions such as ~254, ~269, ~314, ~320, ~336, ~600, ~736 and ~762 in proportion to the TSS. These consensus sequences have been recognized as the binding site for the proteins called CAAT-box binding factors, mainly included in the control of initiation (Zou et al. 2011). Moreover, a typical promoter supports the special cis-acting regulatory elements, as the main components included in transcriptional regulation of a dynamic network of gene activity by combining with TFs to control various biological processes.
in eukaryotic plants (Dey et al. 2015). These factors are included in the suppression, activation, and modulation of different signaling pathways in plant cells under abiotic and biotic stresses. Bioinformatics analysis showed that several special and fundamental elements connected to abiotic stresses and hormone regulations existing in the 5′ flanking region of ObCVOMT and ObEOMT (Tables 2, 3), indicating ObCVOMT and ObEOMT might respond to different environmental conditions.

Light is necessary for photosynthesis, and numerous green tissue-specific promoters comprise light-inducible elements (Zhou et al. 2012). The bioinformatics analysis of ObCVOMT, and ObEOMT promoters showed that they contain E-box, Pc-CMA2c, GATA, ATCT-motif and GT1-motif as the essential cis-elements engaged in the regulation of light-responsive genes (Eulgem et al. 2000; Rushton et al. 2010). Additionally, GT1-box is responsive to light cis-acting regulatory element, which regulates the SA-inducible gene expression level and is cell type-specific (Villain et al. 1996). As Kehi (2006) reported, the GATA-motif is a cis-acting regulatory element necessary for light-regulated, high-level, and tissue-specific expression. It is also conserved in the promoter of whole LHCII type I cab genes (Pietrzykowska et al. 2014). Thus, these motifs can participate in the regulation of the green tissue-specific, and light-induced expression of ObEOMT. The same procedure was seen in the GSE from Oryza rufipogon when light acted as an abiotic stimulus (Xue et al. 2018).

The last cis-acting motifs in ObCVOMT promoter that were found were CGTCA, GT1-box and DOF-box, which may also be participated in regulating the expression of...
ObCVOMT in MeJA, SA, and auxin-inducible responses, although further considerations are required to assess this possibility. Another result from bioinformatics analysis of the ObEOMT promoter is the frequency of cis-acting elements (CGTCA-motif, ABRE, MYC-recognition site, TGACG-motif, TCA-element and PYRIMIDINEBOX-OSRAMY1A) as the most abundant elements concerning hormone responsiveness, which are reported to play essential roles in the MeJA, abscisic acid, gibberellin and salicylic acid (SA; Lopez-Molina and Chua 2000; Zhou 1999; Nakashima et al. 2006). According to these observations, we can infer that these particular cis-acting elements in the promoter of the ObEOMT promoter can have a crucial effect on the exogenous hormone’s response. However, more detailed studies seem necessary to ensure if changes in the transcription level of ObEOMT are dependent on abiotic stress through an ABA-independent pathway. In the growth and development processes of the plant, hormones play a prominent role. Abscisic acid is a broad-spectrum plant hormone involved in signal transduction pathways for abiotic and biotic stresses (Agarwal and Jha 2010). The ABA-independent and ABA-dependent signaling pathways are the main signaling pathways that participated in responding the abiotic stress (Zhu 2002; Yamaguchi-Shinozaki et al.)
2006). Furthermore, the DRE/CRTs (dehydration-responsive elements), ABREs (abscisic acid-responsive elements), or MYC and MYB recognition motifs in different promoters are known to be involved in regulating these pathways and support the responsiveness of genes to stress (Mahajan and Tuteja 2005). Methyl jasmonate, and SA also plant's hormones acting as endogenous signals induce systemic acquired resistance in plants. These hormones specially bind to a variety of plant proteins which affect their activity and regulate the plants response to abiotic and biotic stresses (Wang et al. 2020). In several plants, the up regulations of the transcriptional levels by SA and methyl jasmonate have been recorded for the genes involved in phenylpropanoids pathway, including GbPAL (Zhang et al. 2014), GbANS (Xu et al. 2008b), and GbFLS (Xu et al. 2012).

Also, the ObCVOMT and ObEOMT promoters contain some the putative cis-acting elements including W-box, Myc-core, MBS, CBFHV, LTRECOREATCOR15, DRE and ACGTATER1 sequences owning effectiveness in drought, cold, high salinity, dark-induced senescence and low-temperature stresses (Wang et al. 2019). The conservative binding sites for the two transcription factors, MYC and MYB, are effective in hydration stress, and regulating the signal transduction pathways between gene expression and perception of water-stress signal (Agarwal et al. 2006; Liao et al. 2015). MYB and MYC core elements are known to be involved in the promoter of the Petunia CHSJ, CHI and other structural genes that participated in regulating flavonoid and anthocyanin biosynthesis (Hichri et al. 2011). The MYB-related protein of Arabidopsis plants, AtMYB2, binds the MYB recognition sequence, resulting the higher binding of the MYB-related transcription factor in the regulating the genes which are associated with water stress (Abe et al. 2003). The promoter of the CPS gene (triggered by ABA treatment in Salvia, miltiorrhiza, and dehydration stress) bears MYB recognition sites as cis-acting elements (Szymczyk et al. 2016). Previous studies showed that the MYCCONSENSUSAT box is the target for the R2R3-type MYB transcription factor, which is participated in cold regulation of CBF genes and acquired freezing tolerance (Agarwal et al. 2006). Previous results showed, W-box motif have a significant effect on the response to abiotic stress as an essential component during water deficit stress (Liao et al. 2015).

Using stable expression assays, the activity of the full-length promoters of ObCVOMT and ObEOMT exhibited that the isolated region of these promoters can induce the GUS expression in leaves of transgenic tobacco under water deficit stress levels. Unique dehydration of drought cis-acting elements is correlated with high activity and up-regulation of pObCVOMT to drive the GUS gene in tobacco leaves in response to the water deficit stress levels. Moreover, analysis of qRT-PCR analysis of transformed plants (pOb-CVOMT-GUS) demonstrated that pObCVOMT-directed GUS expression was water deficit stress levels-specific, which may imply the essential and functional ability of pObCVOMT promoter to drive water deficit stress-specific expression.

Based on both histochemical GUS assay and qRT-PCR analysis, we also found that the GUS expression was induced by the 1208-bp pObEOMT promoter sequence in response to the water deficit stress treatments. We noted that treatment with water deficit stress levels (W1, W2 and W3) resulted in GUS activity of the pObCVOMT, and pObEOMT promoters, which was in agreement with the presence of drought-responsive elements. This result indicates that the pObCVOMT and pObEOMT promoter sequences can drive inducible expression and show activity in the tested condition, probably owing to the presence of specific cis-elements engaged in the control of water deficit stress response. The overall knowledge concerning the complicated interactions between TFs and promoters at the transcriptional level is minimal.

Several studies have shown the crucial role of distal promoter regions in stress-related responses (Behnam et al. 2013; Imatiaz et al. 2015). Imatiaz et al. (2015) observed that the 2.7-kb CmBBX24 promoter sequence of Chrysanthemum had activity in Arabidopsis transgenic plants under drought and salt stress and the elimination of the distal promoter fragments resulted in the reduction of the promoter activity. As Conforte et al. (2017) reported, the cis-elements is the essential elements for the response to the abiotic stress and, or dehydration. According to the results, it could be deduced that the specific dehydration or drought stress cis-acting regulatory elements participated in the promoter activity to drive the reporter gene expression, which means that this information will support pObEOMT promoter being a water deficit stress-specific promoter. However, the importance of each sequence of water deficit stress-inducible motifs requires further confirmation. To clarify the regulatory mechanisms of pObEOMT in response to water deficit stress, it would be indispensable to investigate the TFs that connect to pObEOMT promoter under different levels of water deficit stress treatments.

**Conclusions**

We attempted to clone the promoter region of basil ObCVOMT and ObEOMT genes. Subsequently, we identified the cis-acting regulatory elements responding to signaling molecules and abiotic stress according to the PLACE and PlantCARE databases. Moreover, we identified several typical light, drought, low temperature, and hormone-responsive cis-acting regulatory elements. The results of the GUS histochemical staining and qRT-PCR analysis indicated that pObCVOMT and pObEOMT promoters drove the expression of GUS reporter gene in the transgenic tobacco.
leaves and generally functioned as signaling molecule/stress-inducible promoters. The data obtained in this study revealed that these promoters could be used to improve the resources for inducible promoters. These genetically enhance the particular secondary metabolites content in plant tissues and optimize the molecular breeding of basil for greater methyl chavicol and methyl eugenol levels under abiotic stresses.

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Author contributions FKh and MR conceived, designed and performed the experiments; FKh supervised experiments; MR and ZSh analyzed the results; FKh wrote the manuscript.

Declarations

Conflict of interest The authors declare no competing financial interests.

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