Identification and Functional Characterization of the CVOMT and EOMT 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 indicates these promoters are able to drive inducible expression. The *cis*-acting elements analysis showed that the *pObCVOMT* and *pObEOMT* promoters contained dehydration or water deficit-related transcriptional control elements.

1. Introduction

One class of important compounds, volatile phenylpropenes (methyl chavicol and methyl eugenol), make up the essential oils in aromatic and herbaceous plants. These compounds are used in culinary preparations, fragrances and bioactive substances for both plants as herbivores, insect pollinator attractants, antibacterial, antifungal agents and also for human nutrition (Devi et al., 2010). Volatile phenylpropenes are the major constituents of *Ocimum basilicum* (one genus from the Lamiaceae family regarded as the most important medicinal and ornamental plant for 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 and in fragrance and medical industries (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 yet have not been identified in detail. Briefly, the primary steps begin with general phenylpropanoid biosynthetic via 4-coumaric acid, cinnamic acid, ferulic acid and caffeic acid productions (Dixon et al. 2002; Ehlting et al. 2006). The next steps are followed by the subsequent biosynthetic pathways through de-amination, methylation and decarboxylation steps, and adding methoxyl and hydroxyl functional groups (Dixon et al. 2002; Gang et al. 2001). The final step in biosynthesis of phenylpropene, methyl chavicol and methyl eugenol is catalysis by eugenol *O*-methyltransferase (E*O*MT) and chavicol *O*-methyltransferase (CV*O*MT) enzymes (Vogt, 2010).

Owning to these essential roles in plant defense system 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 E*O*MT) and characterized their expression patterns in 3 basil cultivars during water deficit stress treatments (Khakdan et al. 2017). Several studies have also revealed that the expression of *CVOMT* and E*O*MT genes at the metabolite levels may vary in different cultivars, plant drying methods and during drought stress (Pirbalouti et al. 2013; Al-Kateb and Mottram, 2014; Khakdan et al. 2016, 2017).

However, detailed studies on the promoter structure of the *CVOMT* and E*O*MT genes remain elusive. To achieve a high concentration of the distinct metabolites in specific plant tissues and under various environmental conditions including drought, high salinity and strange temperatures, the identification of spatial- and tissue-specific promoters of crucial enzymes under stress-responsive transcription factors (TFs) arises as a key strategy in metabolic engineering approaches (Zhou et al. 2012).

Since the biosynthesis of volatile phenylpropenes, methyl chavicol and methyl eugenol occurs at different growth stages of the *Ocimum* species and these compounds are regulated under abiotic stresses, the control of water stress-tolerant genes expression by tissue-specific and stress-responsive promoters has been applied as an essential alternative strategy to produce these compounds without facing the adverse effects associated with expression of the constitutive gene driven by CaMV 35S promoter in new genetically-modified crops (Tu et al. 2009).

Understanding the transcriptional regulation of the gene encoding rate-limiting enzymes of a particular secondary metabolite pathway could be achieved by controlling the overexpression of particular *trans*-factors of the crucial enzyme genes playing a marked role in regulating such enzymes under particular 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 which remains active under specific environmental conditions or positively responds to the action of special *trans*-factors in the production of special 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).

In spite of tremendous efforts devoted to study the volatile phenylpropanoid biosynthesis pathway, only few TFs which regulate the pathway structural genes expression yet have 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, only RNA interference (RNAi) which inhibited ODO1 expression reflected its ability to particularly regulate 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. In this study, 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 water deficit stress-specific expression patterns of the promoters in the leaves of transgenic tobacco plants were studied using histological analyses.

2. Materials And Methods

2.1. 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 two-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 (*Nicotiana 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 *A. tumefaciens*-mediated method.

2.2. 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 (*Dra*I and *EcoR*V), 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 Adapt-*Dra*I and AP2: Adaptor-1 and Adapt-*Eco*RV) (Table 1). The digested DNA genomic of basil was ligated separately to the adaptors (AP1 and AP2), and 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 No. AB530137 and AF435008 for *ObCVOMT* and *ObEOMT*, respectively) and adaptor-primer.

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

Adaptor/Primer name	Adaptor/primer sequences	Modification
Adapt-1	5'-ACTATAGTGACTGCTGGTCGAGGGCCCGGGCTGGT-3'	-
Adapt-*Dra*I	5'- AACCGACTGCCC – 3'	5'phosphate and 3'H2N
Adapt-*Eco*RV	-AATTACCAGCCC-3"5	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'- ATGGGGATGGATTGGAGTAATTGGGG-3'	-
GSP2- E	5'- TAAGGACATTGAGTTGCAAAGGCGTAC-3'	-
adaptor-primer	5'-ACTGCTGGTCGAGGGC-3'	-
*pObCVOMT-Hin*dIII–F	5'- CCC AAG CTT AAA TGG GTC AAT TTC GGG TTG – 3'	
*pObCVOMT-Bam*HI-R	5'- CGC GGA TCC TGG ATG TAA TTT ATG TGG TAA ACTAGG – 3'	
*pObEOMT-Hin*dIII–F	5'- CCC AAG CTTGAA ATT GTC GGT CCT GGA GAG – 3'	
*pObEOMT- Bam*HI-R	5'- CGC GGA TCC GCT CGG GCT GGT ATC A – 3'	
uidA-F	5'-TGATAGCGCGTGACAAAAA-3'	
uida-R	5'-CGAAATATTCCCGTGCACTT-3'	

Common PCR was performed using a Bio-Rad thermocycler in a final volume of 20 µL, which contained 0.2 mM of dNTPs, 1X 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 glassmilk 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]).

The sequence data was aligned by the BLAST algorithm option available at NCBI (http://www.ncbi.nlm.gov/). The putative transcription start site, *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).

2.3. Construction of the pObCVOMT::GUS expression vector

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

2.4. Tobacco transformation and growth condition

Two plant expression vectors for each promoter, *pObCVOMT::GUS*, *pObEOMT::GUS* and pBI121 (*p35S::GUS*) were introduced into *Agrobacterium tumefaciens* strain C58 by the freeze-thaw method (Sambrook and Russell, 2001). These vectors were subsequently moved into *Agrobacterium tumefaciens* (strain C58) according to the method described by Wang 2006. Briefly, *A. tumefaciens* from an individual colony was grown at 28^{o}C for 48 h in 25 mL of YEP medium (5 g/L Bacto Peptone, 6 g/L yeast extract, 2 mM $MgSO_4$ and 5 and g/L sucrose) supplemented with kanamycin (50 µg/mL) and rifampicin (50 µg/ml) at 28^{o}C. It was then added to 50 mL of induction medium (YEP medium along with 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 cm^2 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 a 8-h dark/16-h light cycle. Ultimately, three transgenic plants for each construct were used for water deficit stress.

2.5. Water deficit stress treatments

Prior to water deficit stress treatment, seedlings of two 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, three stress levels were set based on field capacity (FC) through withholding water and labeled as *W1*: 75% FC, *W2*: 50% FC and *W3*: 25% FC with three replicates. Plant sampling was done after water deficit stress treatment for one week, and for control treatment, the plants were developed with no water deficit stress at the time period similar to water deficit stress.

2.6. 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 X100) 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).

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

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

The leaf total RNA samples of each the three treated transgenic plant (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 agarose gel electrophoresis-based system. The cDNA synthesis reaction was initially performed using the Revert-AidTM 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*) was 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 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 analyze using analysis of variance (ANOVA) to assess the significant differences between treatments using Duncan's multiple range tests ($p \leq 0.05$).

3. Results

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

A 616-bp upstream fragment of *ObCVOMT* gene (Fig. 2a) was isolated from *O. basilicum* genome by the 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 sequence 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 transcription start site (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 (CCAAT) 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. 2016; 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 important 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 hormones responsive were detected (Fig. 3). The CGTCA motif, LTRECOREATCOR15, overlapping with and 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*.

Table 2
Putative *cis*-acting regulatory elements identified in the chavicol *O*-methyltransferase (*ObCVOMT*) promoter sequence, according to the prediction of PLACE and PlantCARE.

Site name	sequence	Number of *Cis*-elements (+) strand (-) strand	Distance from TSS	Function
TATA-box	TATATATA	1 0	-35	Common *cis*-acting element in promoter and enhancer regions
CAAT-box	CCAAT	1 0	-79	Core promoter element for transcription start
CGTCA	CGTCA	3 0	-134, -156, –296	*Cis*-acting regulatory element involved in the MeJA-responsiveness
MBS	GTCAAC	1 0	393-	MYB binding site involved in drought-inducibility and flavonoid biosynthesis
Pc-CMA2c	GCCCACGCA	1 0	-171	Part of a light responsive element
GATA-motif	GATA	0 1	-324	Part of a light- and tissue specific-responsive element
EBOXBNNAPA (E-box)	CANNTG	1 0	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 0	353-	MYC recognition site involved in dehydration and cold responsiveness
CBFHV	GTCGAC	0 1	353-	A dehydration-responsive element (DRE)
LTRECOREATCOR15	CCGAC	1 0	-368	A cold, drought and ABA-responsive element
DRE	ACCGAC	1 0	369-	Dehydration, high salinity and cold responsive element
ACGTATERD1	ACGT	1 0	187-	Involved in upregulation by dehydration stress and dark-induced senescence
W-box	TGACT		-389	WRKY binding site, involved in many physiological processes

Site name	sequence	Number of *Cis*-elements (+) strand (-) strand	Distance from TSS	Function
GT1-Box	GRWAAW	1 3	-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 3	-414, -448, -469, -511	Auxin-responsive element
WRKY-binding site	TGAC	1 4	133, 155, 175, 305, 399	WRKY binding site, involved in many physiological processes

The *ObCVOMT* promoter contains one conservative binding site (MBS motif) identical to the plant MYB transcriptional factors which are 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 E-box and W-box at -353, -389 and − 356, respectively, in the *ObCVOMT* promoter (Fig. 3) as conservative binding motif of MYC, bHLH (Abe et al. 1997; Abe et al. 2003, Wang et al. 2016), 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 that 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 important role in the activation of the biological defense process.

3.2. 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 transcription start site 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.

Table 3
Summary of the Predictions of the cis-elements and Functions identified in 5' flanking region of the eugenol *O*-methyltransferase (*ObEOMT*) using PlantCARE and PLACE database.

Site name	sequence	Number of *Cis*-elements (+) strand (-) strand	Distance from TSS	Function
TATA-box	TATA	2 0	-27, -99,	Common *cis*-acting element in promoter and enhancer regions
	ATATAA	1 0	-410, -523	
	TAAAGATT	0 1		
CAAT-box	CAAAT	1 0	-254, -269,	Core promoter element for transcription start
	CAAT	3 1	-314,	
	CCAAT	2 0	-320,	
	CATTC	1 0	-336, -600,	
	CCAAT	1 0	-736, -762,	
			-858	
ABRE	AACCCGG	0 1	610-	*Cis*-acting element involved in the abscisic acid responsiveness
AE-box	AGAAACAA	0 1	87	Part of a module for light response
ATCT-motif	AATCTAATCC	1 0	797-	Part of a conserved DNA module involved in light responsiveness
CCAAT-box	CAACGG	1 0	313-	MYBHv1 binding site
CGTCA-motif	CGTCA	2 1	-273, -485, -561	*Cis*-acting regulatory element involved in the MeJA-responsiveness
GT1-motif	GGTTAA	1 0	-500	light responsive element
	GGTTAAT	1 0	-501	
MYB recognition site	CCGTTG	0 1	-313	MYB binding site involved in drought-inducibility

Site name	sequence	Number of *Cis*-elements (+) strand (-) strand	Distance from TSS	Function
MYC- recognition site	CATGTG	0 1	-354	Involved in transcriptional control in drought- and abscisic acid-response
TCA-element	TCAGAAGAGG	0 1	508-	*Cis*-acting element involved in salicylic acid responsiveness
TGACG-motif	TGACG	1 1	-273, -561	*Cis*-acting regulatory element involved in the MeJA-responsiveness
LTRE-motif	ACCGACA	0 1	-914	Putative low temperature responsive element
PYRIMIDINEBOXOSRAMY1A	CCTTTT	0 1	-719	Gibberellin-Responsive
W-box	TGACY	0 1	-767	WRKY binding site, involved in many physiological processes
MYCCONSENSUSAT (MYC-core)	CANNTG	4 0	-144, -354, -620, -676	MYC recognition site involved in dehydration and cold responsiveness

The most potential putative TATA box was located at -27 (ATATAA), similar to the majority of other eukaryotic genes (Zou et al. 2011). Moreover, a series of potential regulatory elements which simplify the inducible expression of *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 element 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).

3.3. Tobacco transformation and screening transgenic

To understand the functional roles and activeness of the *pObCVOMT* and *pObEOMT* promoter regions in response to environmental stress, the promoter fusion to the *GUS* reporter gene was established (Fig. 1). As positive and negative controls, *p35S*::*GUS* transgenic tobacco (including pBI121 with 35S promoter construct), and non-transgenic plants were used for a better comparison of expression pattern, respectively. The three resulting plant expression constructs, the *pObCVOMT*::*GUS* and *pObEOMT*::*GUS*, were used 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.

3.4. 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 and 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 strong GUS activity. As regards 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 strong staining was observed. However, when transgenic plants grew under *W1* and *W2* water deficit stress levels, *in situ* GUS staining was detected (Fig. 5B). However, *pObEOMT* was unable to drive the expression of *GUS* gene with different expression levels with *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 density of water deficit stress (Fig. 5B). We infer that the presence of maximum number of MYC-recognition site as the main dehydration-responsive elements also play an important role in the response of water deficit stress and may elevate high levels of *uidA* expression under *W3* (5.12-fold). As expected, activity of GUS in positive control tobacco was transformed with Agrobacterium-harboring *p35S::GUS* construct, showing a robust activity, whereas no GUS activity was found in non-transgenic tobacco (negative control) (Fig. 5B and C).

4. Discussion

Promoters are essential elements that regulate gene expression both temporally and spatially and induce transcription (Juven-Gershon and Kadonaga 2010). Most of the 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 in order 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 specific plant tissues or at specific times (Zhou et al. 2012). Therefore, application of very specific 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 greater accumulation of a particular secondary metabolite. Avoiding continuous and high concentrations of desired products especially compounds, like toxins, may interact with other metabolic pathways and induce destructive effects in transgenic plants (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 phenylpropenes, 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 though which the *ObCVOMT* and *ObEOMT* genes expression is regulated when basil is subjected to water deficit stress, we initially isolated 616 bp- and 1,208-bp long promoters of *ObCVOMT* and *ObEOMT*, respectively (Fig. 2) and determined numerous *cis*-regulatory elements in them. These elements are important molecular switches in the *ObCVOMT* and *ObEOMT* genes' regulatory networks, according to the PLACE and PlantCARE databases (Figs. 3 and 4, Tables 2 and 3). Consequently, we functionally analyzed the *ObCVOMT* and *ObEOMT* promoters in order to determine the role of the predicted main regions and the activity of the full-length isolated promoter influencing *GUS* gene expression during 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 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 accurate start of the transcription process by RNA polymerase, which is often presumed to be a special region with widespread 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 & 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 transcription start site. These consensus sequences have been recognized as the binding site for the proteins called CAAT-box binding factors, which are mainly included in 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 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 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 5' flanking region of *pObCVOMT* and *pObEOMT* (Tables 2 & 3), indicating *ObCVOMT* and *ObEOMT* might respond to different environmental conditions.

Light is necessary in 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), which can interact specially with a family of bZIP proteins, suggesting light as a significant regulator for *ObCVOMT* and *ObEOMT* expression. The E-box was a *cis*-acting element and perceived by BZIP, R2R3-MYB, and BHLH factors which are important for controlling the tissue-specific and light-responsive activation of phenylpropanoid biosynthesis genes (Hartmann et al., 2005). Additionally, GT1-box is a responsive to light *cis*-acting regulatory element which regulates the SA-inducible gene expression level and is cell type-specific (Villain et. 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 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 be also 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 PYRIMIDINEBOXOSRAMY1A) as the most abundant elements in relation to hormone responsiveness, which are reported to play important

roles in the MeJA, abscisic acid, gibberellin and salicylic acid (Lopez-Molina and Chua 2000; Zhou 1999; Nakashima et al. 2006). According to these observations, we can infer that these special *cis*-acting elements in the promoter of *ObEOMT* promoter can have a key 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 participated in responding the abiotic stress (Zhu, 2002; Yamaguchi-Shinozaki et al. 2006), the DRE/CRTs (dehydration-responsive elements), ABREs (abscisic acid-responsive elements), or MYC and MYB recognition motifs in different promoters are known to be involve in regulating these pathways and support the responsiveness of genes to stress (Mahajan and Tuteja 2005). Methyl jasmonate and salicylic acid (SA) are also plant hormones acting as endogenous signals which 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, countless up regulations of the transcriptional levels by SA and methyl jasmonate have been recorded for the genes engaged in phenylpropanoids, including *GbPAL* (Zhang et al. 2014), *GbANS* (Xu et al. 2008b) and *GbFLS* (Xu et al. 2012).

Another characteristic of *ObCVOMT* and *ObEOMT* promoters is that they contain some putative *cis*-acting elements including W-box, Myc-core, MBS, CBFHV, LTRECOREATCOR15, DRE and ACGTATERD1 sequences, which are effective in responses to 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 considered to be 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 participated in regulating flavonoid and anthocyanin biosynthesis (Hichri et al. 2011). The MYB-related protein of Arabidopsis plants, AtMYB2, bind the MYB recognition sequence and the MYB-related transcription factor engaged in 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 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). The motif W-box can have a significant effect on the response to abiotic stress and may function as an important component during water deficit stress (Liao et al. 2015).

Using stable expression assays, 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. The presence of special dehydration of drought *cis*-acting elements is correlated with high activity and an up regulation of *pObCVOMT* to drive *GUS* gene in tobacco leaves in response to the water deficit stress levels. Moreover, analysis of qRT-PCR analysis of

transformed plants (*pObCVOMT*-GUS) clearly demonstrated that *pObCVOMT*-directed *GUS* expression was water deficit stress levels-specific, which may imply the basic 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 1,208-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. Totally, this result indicates that the *pObCVOMT* and *pObEOMT* promoter sequences are able to drive inducible expression and show activity in the tested condition, probably owing to the presence of specific *cis*-elements engaged in control of water deficit stress response. Currently, the overall knowledge concerning the complicated interactions between TFs and promoters at the transcriptional level is very limited.

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 at the presence of drought and salt stress, but the elimination of distal promoter fragments resulted in the reduction of promoter activity under drought stress. Conforte et al. (2017) reported that the existence of these *cis*-elements was related to the response to abiotic stress and/or dehydration. These findings reveal that the specific *cis*-acting regulatory elements that are induced by dehydration or drought stress may be participated in the promoter activity to drive 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 require further confirmation. To clarify the regulatory mechanisms of *pObEOMT* in response to water deficit stress, it would be highly necessary 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 and subsequently identified the *cis*-acting regulatory elements responding 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 *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 promoter, 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.

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

Declarations

Conflicts of interest/Competing interests

The authors declare no competing financial interests

Authors' contributions

F.K. and M.R. conceived the experiments; F.K. supervised experiments; M.R. and Z.Sh. analyzed the results; F.K. wrote the manuscript.

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Figures

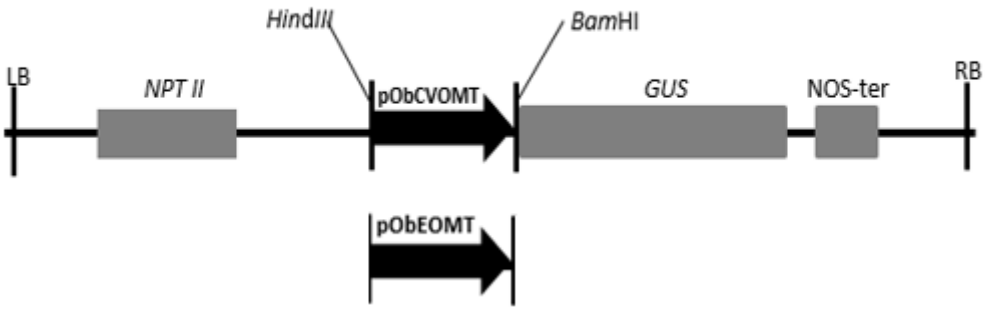

Figure 1

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, GUS gene is placed under the control of pObCVOMT and pObEOMT promoters, instead of CaMV 35S promoter. LB left border, NPTII neomycin phosphotransferase II, GUS (β-glucuronidase) reporter gene, NOS nopaline synthase terminator; RB right border.

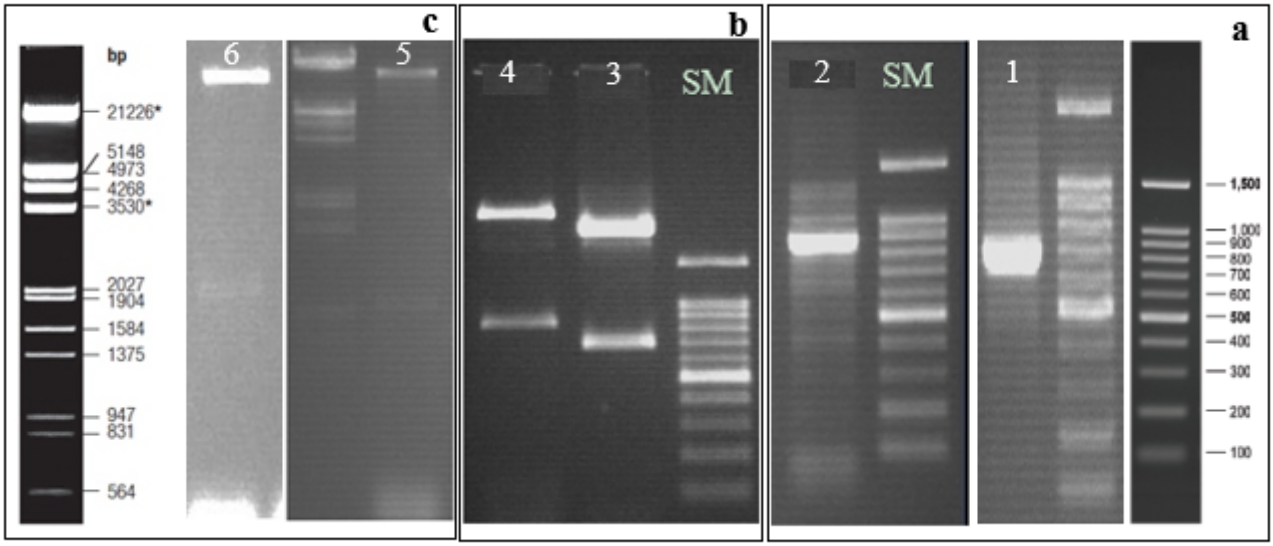

Figure 2

Confirmation of isolation and cloning of pObCVOMTs and pObEOMTs genes promoters. a) Electrophoresis of PCR product. PCR was performed with specific primers gene specific primer 1 (GSP1-C, GSP1-E for ObCVOMT and ObEOMT, respectively) and adaptor-primer for pObCVOMTs and pObEOMTs gene promoter isolation. Lane 1 and 2; PCR product., Lane M, DNA size marker, 100 bp, Fermentas. b) Cloning confirmation of pEOMTs DNA fragment in pTG19-T vector. Cloning of pObCVOMTs and pObEOMTs DNA fragment, produced by PCR, was confirmed using of enzymatic digestion of recombinant vectors by BamHI restriction enzyme. Lane 3 and 4: pObCVOMTs and pObEOMTs fragments, respectively; M: DNA size marker, 100 bp, Fermentas. c) Confirmation of isolated pObEOMTs cloning in pBI121 vector. Enzymatic digestion of recombinant pBI121 vector confirmed replacement of CamV35S promoter by isolated pObCVOMTs and pObEOMTs fragments in this vector. Lane M: DNA size marker, Lambda. Lane 5 and 6: pObCVOMTs and pObEOMTs fragments, respectively.

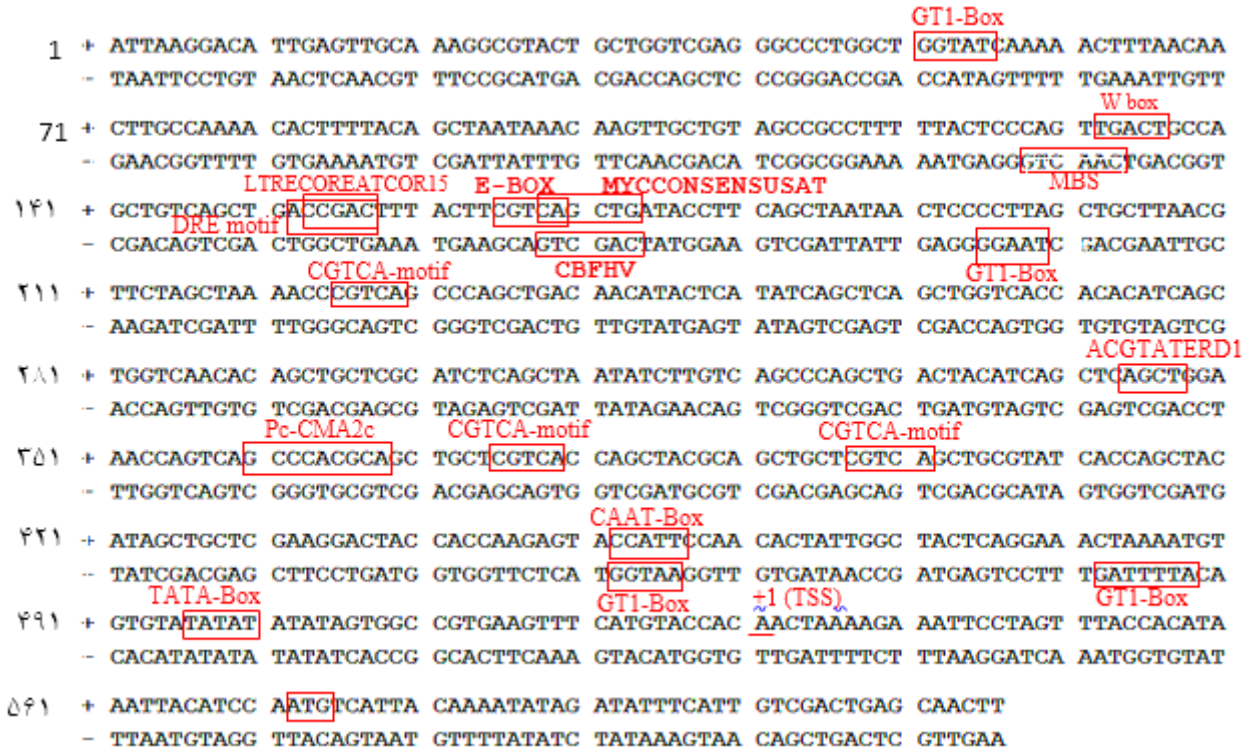

Figure 3

Nucleotide sequence and putative cis-acting elements of the promoter region of the ObCVOMT gene. The transcription start site is underlined. Potentially motifs with similarly to previously identified cis-acting elements (predicted by PLACE and PlantCARE software) and translation initiation codon (ATG) displayed in a box.

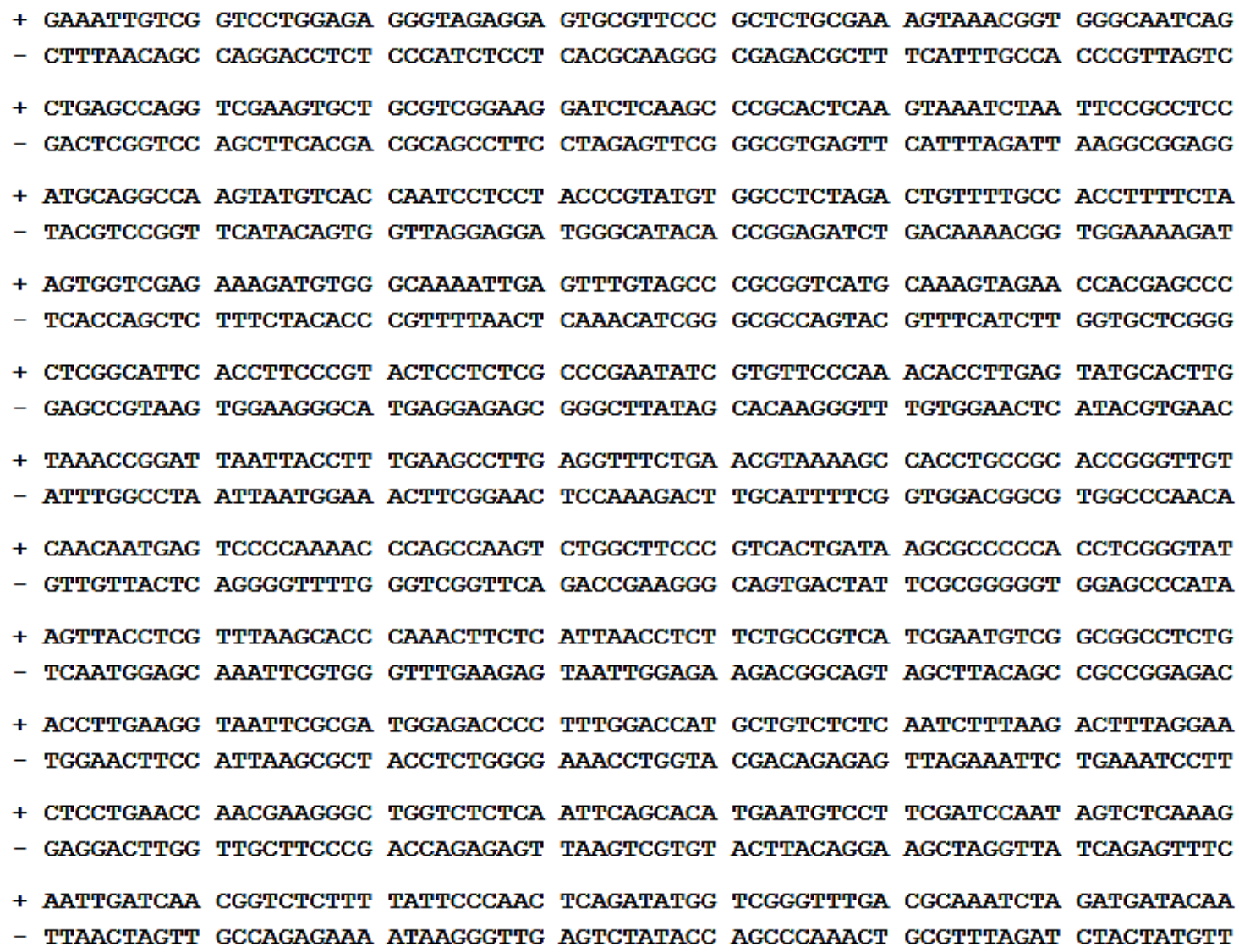

Figure 4

The 5' flanking sequence of ObEOMT. “+1” indicates the predicated transcription start site is underlined. Potentially functional elements predicted by PLACE and PlantCARE software are boxed.

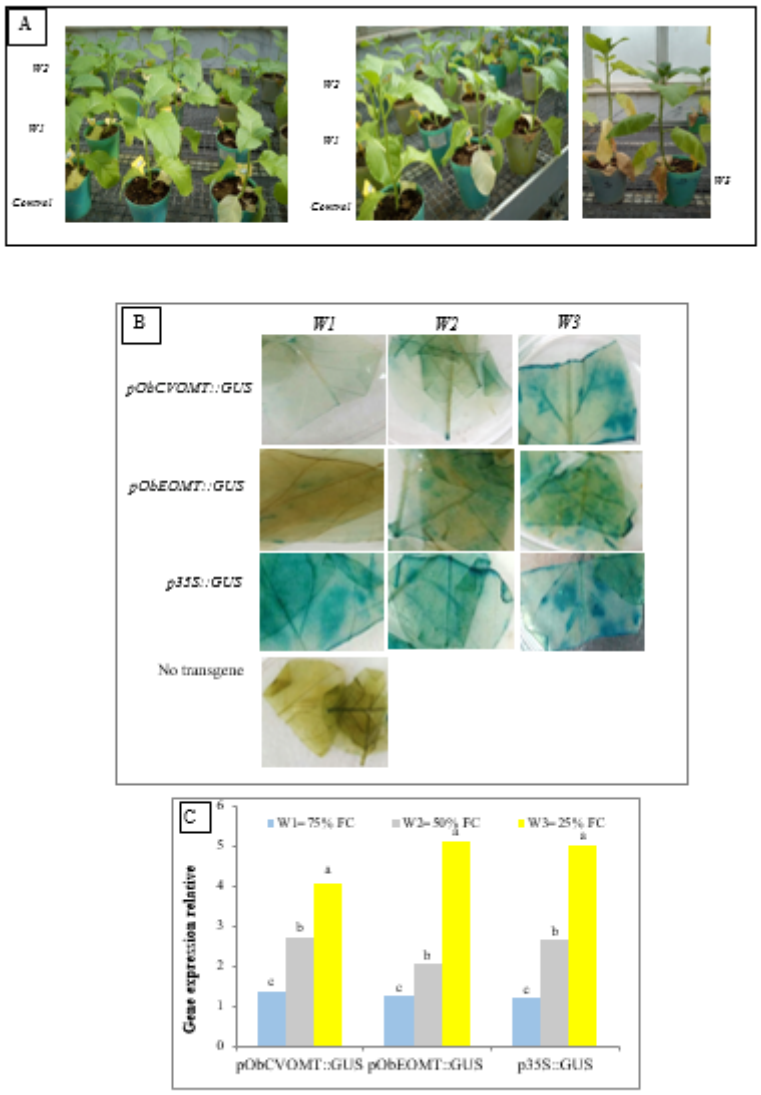

Figure 5

Effects of water deficit stress and Histochemical analysis and differential expression levels of the GUS reporter pObCVOMT, pObEOMT and p35S. A) Effects of water deficit stress on transgenic tobacco seedling carrying pObCVOMT::GUS, pObEOMT::GUS. B) The leaves sampled from transgenic tobacco seedling carrying pObCVOMT::GUS, pObEOMT::GUS and p35S::GUS and no transgenic tobacco under different treatments of water deficit stress (W1, W2, W3 are 75, 50 and 25% FC, respectively) and normal condition. C) The relative expression levels of uidA mRNA in leaves under W1, W2 and W3 water deficit stress levels (75, 50 and 25% FC, respectively). Different letters (a, b, c) indicated above the bar represent statistically significant difference at $p \leq 0.05$ (Duncan's multiple range test).