Research Article

Bioactive Constituent and *Eugenol Synthase 1* Gene of Thai Red Holy Basil (*Ocimum tenuiflorum* L.)

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Abstract
Red holy basil (*Ocimum tenuiflorum* L.) is a commonly grown herb crop with diverse cultivars/accessions that contain highly ranked bioactive substances for medicinal potentialities. In this study, bioactive constituents and *eugenol synthase 1* (*EGS1*) were characterized in Thai red holy basil. The compositions of the bioactive distilled from dried and fresh leaves and dried flower spikes were determined using headspace–solid phase microextraction–gas chromatography. Furthermore, a full-length of putative *EGS1* was cloned from Thai red holy basil leaf tissue. The open reading frame of *EGS1* contained 945 bp and encoded a 314-amino acid sequence. Phylogenetic analysis clearly distinguished two homology classes of EGS. EGS1 of red holy basil was closely related to that of white holy basil and lemon basil. Additionally, *EGS1* expression in leaf tissue at the flowering stage was further assessed in ten local red holy basil accessions. *EGS1* transcript levels were high, especially in aroma-rich accessions. *EGS1* was expressed at higher levels in leaves than in flower spikes. Our study characterizes red holy basil in terms of both biochemical and gene information. This information can be used for further studies focusing on gene editing to increase the production of high-quality Thai red holy basil for the food and pharmacological industries.

Keywords: Bioactive substances, Eugenol, Eugenol synthase 1, *EGS1*, Red holy basil

1 Introduction

Red holy basil (*Ocimum tenuiflorum* L.) is native to the Asian tropics and belongs to the family Lamiaceae. It is among the most well-known species for its medicinal properties and is an important economic and industrial aromatic substance for flavor and fragrance in the food, pharmaceutical, cosmetic, and aromatherapy sectors [1].

The leaves and flowers of red holy basil contain many phytochemicals, the majority of which are phenylpropanoids and terpenoids containing various medicinal and aromatic properties [2]. The major biological active constituent of these phytochemicals is eugenol (4-allyl-2-methoxyphenol). Eugenol is a phenolic compound from the class of phenylpropanoids that is sequestered under specialized physiological conditions, such as in red-violet leaves of red holy basil. Bioactive eugenol is dominant in the 'Green' and 'Purple' varieties (67.4 and 72.8%, respectively) [3] of Asian, North African, Russian, and Eastern European holy basil [4] and acts as a floral attractant of pollinators [5] and as a defense compound against pathogens and herbivores [5], [6]. It has also shown to be imperative in human health, such as exhibiting antibacterial, neuro-protective, larvicidal, antidiabetic, anti-anaphylactic, radioprotective, wound-healing, cardio-protective, anti-genotoxic, antihistaminic, and mast cell stabilization activities [7]–[14].

Bioactive eugenol is synthesized from coniferyl acetate by eugenol synthase 1 (EGS1), the first protein reported to have EGS activity [5]. *EGS1* was previously identified in the EST collections of various eugenol-rich *O. tenuiflorum* varieties [15]. Although EGSs have been characterized and cloned from *Ocimum* species [16] and a few other plants, such as rose (*RcEGS1*) [17] and petunia (*PhIGS1*) [5], information on whether Thai red holy basil plants have distinct *EGS1* and eugenol is still limited.

To gain more comprehensive knowledge about the key genes and bioactive substances in Thai red holy basil, we cloned and analyzed the expression of the *EGS1* gene. Furthermore, we used biochemical identification approaches to identify the bioactive fractions of Thai red holy basil. The aims of this study were 1) to analyze the constituents of bioactive from various tissues, such as fresh and dry leaves and dry flowers, of Thai red holy basil and compare them to those of white holy basil using headspace–solid phase microextraction–gas chromatography (HS-SPME), 2) to isolate and functionally characterize *EGS1* in Thai red holy basil, and 3) to examine the expression of *EGS1*, a precursor enzyme of eugenol biosynthesis, in leaf and flower tissues of various Thai red holy basil accessions.

2 Materials and Methods

2.1 *Plant materials*

Plant tissues were collected from 3-month-old *O. tenuiflorum* L. grown in a greenhouse at the Tropical Vegetable Research Center, Kasetsart University Kamphaeng Saen Campus (Nakhon Pathom, Thailand). The experiment was performed under natural light conditions (32–37 °C day/23–28 °C night temperature; 50–70% day/70–90% night relative humidity).

For HS-SPME, fresh and dry leaves and dry flower spikes of red and white holy basil from the same source were collected in December 2021. Leaf and flower samples were dried in natural air for 48 h and remained on ice following collection and throughout transfer to the laboratory. Then, 1.0 g of dry flower spike or fresh or dry leaf material was placed in a 4 mL clear vial and sealed with a polypropylene open-top cap and a PTFE/silicon septum (Thermo Scientific, Austin, TX, USA) and used immediately.

For gene cloning and expression analysis, leaf tissues were frozen in liquid nitrogen and stored at –80 °C until analysis.

2.2 *Estimation of eugenol and methyl eugenol content*

2.2.1 Extraction of bioactive constitutes by HS-SPME

Extraction of the bioactive constituents from each material was performed using a HS-SPME approach with the modified method of Yamani *et al.* [12]. In accordance with the manufacturer's instructions, an 85 mm polyacrylate fiber attached to a sampling holder (Supelco, Bellefonte, PA, USA) was conditioned for 30 min at 250 °C in the injection port of a gas chromatograph (GC) before use. The polyacrylate fiber was cooled before being injected into the headspace of the sample vial, and the entire system was placed in a heating block at 90 °C for 10 min using a TriPlus RSH autosampler (Thermo Scientific, Waltham, USA). The volatiles were subsequently desorbed for 10 min by inserting the fiber into the GC injection port.

2.2.2 Gas Chromatography-Mass Spectrophotometry (GC-MS/MS)

Identification of the bioactive compounds was performed

using GC-MS/MS (Trace 1310, TSQ 9000, Thermo Scientific). The system was fitted with a TG-5SIL MS with dimensions 30 mm × 0.25 mm, i.d., film thickness 0.25 μm (Thermo Scientific). Helium, as the carrier gas, with a purity of 99.99% was employed at a flow rate of 1 mL/min. The split ratio was 1:10. The initial GC oven temperature was 40 °C for 1 min, followed by a 5 °C/min increase to 250 °C for 1 min. The injection port, transfer line, and source temperatures were heated to 200 °C. The mass scan range was 33–550 m/z. The NIST mass spectral library (Chromeleon 7.2 CDS Software, Thermo Scientific) was utilized for data acquisition and processing. After dividing the peak areas of detected compounds by the total peak area of all identified compounds, a normalized amount of each compound was calculated and expressed as a percentage.

2.3 *EGS1 cloning and identification*

2.3.1 PCR cloning of EGS1 from red holy basil

The gene encoding known putative basil *EGS1* (Accession no. DQ372812), was characterized by Koeduka *et al.* [5]. It was used to design primers for gene cloning. PCR primers (*EGS1*-F: 5' – ATGGA GGAAAATGGGATGAAAAGCA – 3'; *EGS1*-R: 5' – TTAAAATGCTGAAGCCGGCGGTGGAG – 3') targeting the start and stop regions of the coding sequence were designed to amplify *EGS1* from Thai red holy basil. PCR cloning was performed using genomic DNA as the template and Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) using the following PCR conditions: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 3 min; and a final extension at 72 °C for 10 min. PCR amplicons were separated by agarose gel electrophoresis, purified using a QIAGEN Gel Extraction kit (QIAGEN, Valencia, CA, USA), and cloned into pGEM®T Easy Vector (Promega, Madison, WI, USA), after which Sanger sequencing (Macrogen, Seoul, Republic of Korea) was investigated. BLAST nucleotide sequence [18] and EXPASY translated amino acid sequence [19] searches were performed. The sequence was deposited in NCBI GenBank (OQ290802).

2.3.2 Bioinformatics

NCBI's Blast searches were conducted to analyze homologous sequence alignments. The open reading frame for the *EGS1* gene was analyzed using ORF Finder. Sequence alignments were generated using CLUSTALW. A phylogenetic tree was built to analyze the relationships among Thai red holy basil and related species, using the UPGMA method [20]. The bootstrap consensus tree inferred from 1000 replicates [21] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [21]. The evolutionary distances were computed using the p-distance method [22] and are in the units of the number of amino acid differences per site. This analysis involved 12 amino acid sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 314 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [23].

2.3.3 Total RNA extraction and cDNA synthesis

Leaves from ten different red holy basil accessions from Thai genetic sources, named OS001 to OS010, were used to validate *EGS1* expression between different accessions. Meanwhile, *EGS1* gene expression in different tissues, such as leaves and flower spikes, was analyzed in the OS011 and OS012 accessions. Red holy basil accessions were collected from various areas in Thailand and grown in the greenhouse at the Tropical Vegetable Research Center, Kamphaeng Saen, Nakhon Pathom, Thailand.

Total RNA was extracted from the samples using TRIzol reagent (Invitrogen). DNase-treated RNA (4-10 μg) was synthesized into cDNA using SuperScript™ III reverse-transcriptase (Thermo Scientific).

2.3.4 Gene expression analysis

Quantitative reverse transcription-PCR (qRT-PCR) was performed for *EGS1* in 10 μL reactions consisting of 5 μL of 2X SYBR supermix (BioRad, Hercules, CA, USA), 1 μL each of 2.5 mM forward and reverse primers, and 1 μL of cDNA adjusted with nuclease-free water. The *actin1* gene of *O. tenuiflorum* was utilized as the endogenous control, and three biological

replicates of each reaction were conducted using a CFX96 real-time PCR machine (BioRad). Specific primer sequences were designed as presented in Table 1. The annealing temperature was set at 50 °C and the remaining conditions followed the manufacturer's instructions. qRT-PCR was performed for 35 cycles and the products were validated by gel electrophoresis. The relative gene expression levels were calculated using the ΔCt method [24].

Table 1: Primer sequences for qRT-PCR.

Gene	Sequence (5'→3')	Tm (°C)
EGS1	TGGAGGGACAGGTTACATAG (F)	53.0
	GGCTGTCTTGGTGGAATTAG (R)	53.0
Actin1	GTGCGACGTGGATATCAGGA (F)	55.5
	GAGCCTCCGATCCAGACACT (R)	55.5

2.4 *Statistical analysis*

Three biological and three technical replicates for gene expression analysis were conducted. Data are presented as mean±standard error. For gene expression analysis, significant differences were determined using one-way ANOVA test by IBM SPSS ver. 22 (IBM Corp., Armonk, NY, USA), and the mean values were compared by Duncan's multiple range tests at a 95% confidence level.

3 Results and Discussion

3.1 *Distribution of eugenol, methyl eugenol, and other bioactive constituents in the leaves and flower spikes of red holy basil*

Red holy basil is a medicinally important herb that has a range of secondary metabolites important in the food, cosmetic, and pharmacological industries. Constant demand from these industries has led to the development of plant breeding projects utilizing local Thai accessions. The levels of these bioactive constituents are therefore important for red holy basil selection.

Component analysis of the leaves and dry flowers of red and white holy basil by HS-SPME and GC-MS/MS resulted in the identification of 27 and 32 components, respectively (Table 2 and 3). Both red and white holy basil contained predominantly monoterpenes and sesquiterpenes; monoterpenes included camphene and α- and β-pinene, and sesquiterpenes included caryophyllene. These bioactive compounds were identified in all tissues of the tested samples but were present at varying levels as shown in Tables 2 and 3. For example, caryophyllene was detected in red holy basil at different concentrations in the flowers (20.07%), fresh leaves (16.57%), and dry leaves (11.47%) (Table 3) compared to in white holy basil where it was detected in flowers (4.42%), fresh leaves (16.84%), and dry leaves (20.11%) (Table 2). In addition, the borane-methyl sulfide complex was prevalent in white holy basil, with slightly different concentrations in the flowers (21.83%), fresh leaves (20.7%), and dry leaves (17.28%) (Table 2). However, this compound was found mainly in the flowers of red holy basil in small amounts (1.72%). Many bioactive constituents present in white holy basil were minor compounds in red holy basil, such as hexanol, o-cymene, α-terpinolene, linalool, and borneol. These results indicate that the composition of bioactive compounds in red holy basil differs from that of white holy basil.

Interestingly, eugenol was identified as the main active constituent detected in red holy basil, while methyl eugenol was isolated from white holy basil (Tables 2 and 3). In red holy basil, eugenol content varied from 1.46% in flowers to 2.25% and 3.79% in fresh and dry weights of leaves, respectively (Table 3). In the case of white holy basil, eugenol was not detected in any tissues, whereas methyl eugenol was mainly detected in white holy basil; the content was highest in fresh leaves (3.71%) (Table 2).

Eugenol and methyl eugenol have been identified in various chemotypes of holy basil from Thailand [25], [26] and India [27], [28]; these differences in eugenol and methyl eugenol content and composition contributed to the design of this study. These findings could be related to the geographical origin of plants, the season of collection, the stage of development, and/or the method of extraction and analysis. For example, eugenol has been reported to increase under conditions of mild water stress, higher temperatures, and higher light intensity [29]. At the field, water capacity of 75% resulted in the highest eugenol content for *Ocimum* species [30]. The amount of eugenol produced in roots of aseptically grown sweet basil (*O. basilicum*) was much lower than the amount produced in roots of soil-grown plants [31]. The increment of eugenol may be due to the different external factors and stress

conditions on enzyme activity and metabolism caused by tolerance reactions in plants [32]. Additionally, decreases in methyl eugenol content were observed in plants under cold and drought conditions, where cold treatment was induced at 4 °C [29]. Regardless of these factors, only a few bioactive components, namely eugenol and methyl eugenol, are responsible for the main bioactivity of holy basil cultivars or chemotypes. The presence of eugenol and β-caryophyllene in red holy basil is largely responsible for its pharmacological properties as well as its desirability in the food industry. Meanwhile, methyl eugenol, which was found in white holy basil, is an aromatic compound that is used to add flavour to food and scent to perfumes and detergents.

3.2 *Cloning and sequencing analysis of EGS1 from Thai red holy basil*

To identify the homolog(s) of *EGS1* in Thai red holy basil, we amplified red holy basil *EGS1* via PCR. One full-length clone of the *EGS1* gene was detected with a length of 2957 bp containing five exons and four introns (Figure 1) comprising a full open reading frame of 945 bp that translated to a 314-amino acid protein. Exons were 180, 135, 229, 204, and 197 bp in length as indicated by red boxes; introns are

Table 2: Chemical constituents of various white holy basil *O. tenuiflorum* L. tissues at the flowering stage

No.	Compound	Retention Time (min)	Area (%)		
			Fresh Leaves	Dry Leaves	Flower
1	l-Alanine ethylamide, (S)-	1.54	*	*	5.87
2	Fluoroethyne	1.55	7.64	4.46	*
3	Borane-methyl sulfide complex	1.81	20.70	17.28	21.83
4	Propanal, 2-methyl-	1.94	2.15	8.16	2.97
5	Acetic acid ethenyl ester	2.1	*	1.08	*
6	Butanal, 3-methyl-	2.57	1.76	5.97	2.85
7	Butanal, 2-methyl-	2.67	1.57	3.5	2.6
8	1-Penten-3-ol	2.85	*	0.49	*
9	Furan, 2-ethyl-	3.02	*	1.17	*
10	Hexanal	4.69	*	0.4	*
11	2-Hexenal	5.91	*	0.35	*
12	1-Butanol, 2-methyl-, acetate	6.55	*	0.45	*
13	Tricyclo[2.2.1.0(2,6)]heptane, 1,7,7-trimethyl-	7.75	*	0.35	0.66
14	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	7.85	*	0.36	0.25
15	α-Pinene	8.07	9.77	10.01	17.31
16	Camphene	8.54	9.12	7.47	15.82
17	Sabinene	9.2	1.77	1.38	2.92
18	ß-Pinene	9.35	5.00	4.05	7.84
19	ß-Pinene	9.69	0.39	0.4	0.49
20	Octanal	10.09	*	0.35	*
21	o-Cymene	10.74	*	0.41	*
22	D-Limonene	10.89	1.60	1.05	2.43
23	Eucalyptol	10.99	1.54	0.59	1.27
24	γ-terpinene	11.76	0.40	0.89	0.57
25	α-Terpinolene	12.58	*	*	0.34
26	Linalool	12.99	*	*	0.4
27	endo-Borneol	15.15	*	*	0.81
28	Copaene	20.85	2.14	*	0.81
29	Methyleugenol	21.37	3.71	3.36	2.17
30	Caryophyllene	22	16.84	20.11	4.42
31	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	22.9	0.48	0.61	*
32	ß-Copaene	23.53	1.45	*	0.77

* Indicates not detected.

Table 3: Chemical constituents of various red holy basil *O. tenuiflorum* L. tissues at the flowering stage

No.	Compound	Retention Time (min)	Area (%)		
			Fresh Leaves	Dry Leaves	Flower
1	l-Alanine ethylamide, (S)-	1.54	8.8	*	*
2	Fluoroethyne	1.55	4.06	3.71	7.44
3	Pentanal, 2,3-dimethyl-	1.74	*	*	0.62
4	Borane-methyl sulfide complex	1.81	*	*	1.72
5	Carbon disulfide	1.9	5.58	*	*
6	Propanal, 2-methyl-	1.94	*	*	0.97
7	Butanal, 3-methyl-	2.57	*	*	1.23
8	Butanal, 2-methyl-	2.67	0.43	2.22	0.77
9	Heptane	3.03	*	*	0.71
10	2-Hexenal	5.91	0.37	*	*
11	Furan, 2,5-diethyltetrahydro-	7.04	0.79	0.49	*
12	Tricyclo[2.2.1.0(2,6)]heptane, 1,7,7-trimethyl-	7.75	0.65	0.75	*
13	α-Pinene	8.07	17.51	18.99	7.71
14	Camphene	8.54	14.81	15.23	7.13
15	Sabinene	9.2	2.39	2.83	1.65
16	ß-Pinene	9.35	7.69	8.61	4.83
17	ß-Pinene	9.69	0.81	0.73	0.8
18	Octanal	10.09	*	*	7.22
19	D-Limonene	10.89	2.31	2.14	1.62
20	Eucalyptol	10.99	3.25	3.37	8.69
21	Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-	11.08	*	*	1.24
22	ß-Ocimene	11.40	2.37	2.3	12.57
23	Linalool	12.99	0.98	0.67	2.47
24	endo-Borneol	15.15	0.85	0.48	1.02
25	Eugenol	20.12	2.25	3.79	1.46
26	Caryophyllene	22	16.57	11.47	20.07
27	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	22.9	0.46	0.44	0.64

* Indicates not detected.

represented as connective lines (Figure 1). The sequence was deposited in NCBI's database with the accession number OQ290802. The amino acid sequences encoded by *EGS1* were 96–99% homologous with eugenol synthase from *O. tenuiflorum* (AOC97444.1), *O. americanum* (AOC97450.1), and *O. gratissimum* (AOC97442.1).

The sequence of the *EGS* gene from *O. americanum* L. has been characterized and is homologous with *EGS1* from red holy basil as they both have a sequence length of 945 bp and encode a 314-amino acid protein with an estimated molecular weight of 36 kDa. The conserved lead residue Lys132 was detected in all EGSs. The tertiary complex structure of EGS binds the cofactor $NADP^+$. The catalytic site of EGS involves the conserved Lys132, which, when prepared for phenylalanine, is the precursor of eugenol in the phenylpropanoid biosynthesis pathway [33], [34].

Figure 1: Structure of Thai red holy basil *EGS1*. Exons are presented as red boxes and introns as connective lines.

To study the evolutionary relationship between EGS1 amino acid sequences and other EGS proteins from different plant species, we constructed phylogenetic trees that indicate that red holy basil EGS1 is closely related to white holy basil *O. tenuiflorum* and lemon basil *O. americanum* EGS1 (Figure 2). Two monophyletic clades were identified. The first clade included EGS1 from Thai red holy basil and EGS from *O. tenuiflorum* and *O. americanum*. The second clade included the fully sequenced EGS from basil *O. basilicum*,

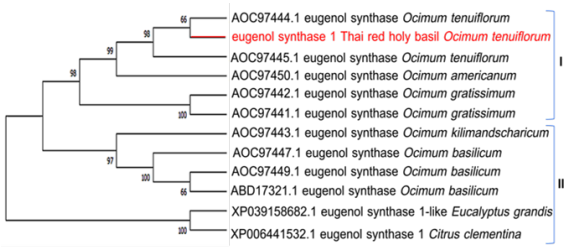

Figure 2: Phylogenetic tree based on amino acid sequences of eugenol synthase. Eugenol synthase 1 of Thai red holy basil is represented in red.

camphor basil *O. kilimandscharicum*, and African basil *O. gratissimum* and EGS1 from the other genuses as the outgroup (Figure 2). Within clade II, *O. gratissimum* differed from the other *Ocimum* we analyzed.

High genetic dissimilarity was observed between *Ocimum* species, demonstrating that the level of genetic divergence within the species is substantial and suggesting that the genetic base is quite broad. Figure 2 illustrates the grouping of *O. tenuiflorum* into the same group as *O. americanum* which is 'Sanctum', while *O. basilicum*, *O. kilimandscharicum*, and *O. gratissimum* were placed in the 'Basilicum' group. In previous reports, based on morphological and phytochemical characteristics, various species have been divided into two groups – 'Basilicum' and 'Sanctum' [35]. Thus, the genetic diversity estimation among the six *Ocimum* species under investigation suggests that phylogenetically *O. tenuiflorum* and *O. americanum* are more closely related to Thai red holy basil than the other four species. The EGS sequences allowed the classification of Thai red holy basil and other *Ocimum* plants.

3.3 *Expression analysis of EGS1 in different tissues and accessions of Thai red holy basil*

To analyze *EGS1* gene expression in eugenol-enriched tissues of red holy basil, qRT-PCR was performed to detect *EGS1* transcriptional levels in leaves and flowers of two accessions of local Thai red holy basil (OS011 and OS012) (Figure 3). The expression of *EGS1* was also analyzed in ten different accessions of local red holy basil (OS01 to OS10) as shown in Figure 4.

EGS1 expression analysis of OS011 and OS012 indicated significantly ($P < 0.05$) higher transcript

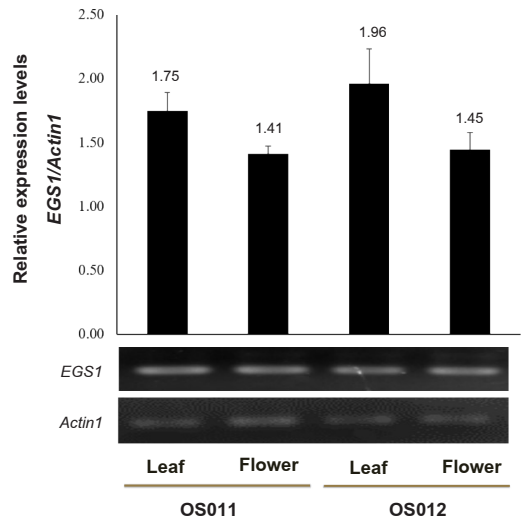

Figure 3: *EGS1* transcript expression in leaves and flowers of two different accessions of Thai red holy basil. Error bars represent standard error ($n = 3$).

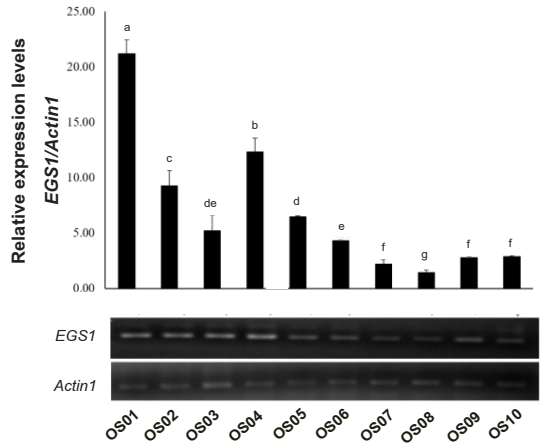

Figure 4: Relative *EGS1* transcript expression in the leaves of ten accessions of Thai red holy basil at the flowering stage. The different letters above the bars represent the significant difference among means ($P < 0.05$). Error bars represent standard error ($n = 3$).

expression in leaves than in flowers (Figure 3). These results are consistent with the *EGS1* expression levels in clove (*Syzigium aromaticum* L.) that were higher detected in young leaves and buds than in flowers of the mature plants. In addition, the expression of *EGS1* in sweet basil is also highly expressed in the specialized organ of the leaf known as peltate

glandular trichomes [31]. The *EGS1* transcript levels in red holy basil additionally showed a correlation with higher eugenol accumulation in leaves than in flowers (Table 3). Interestingly, previous studies also have demonstrated the role of *EGS1* to produce eugenol from coniferyl acetate and their strong correlation to amount of eugenol synthesized [36]. Therefore, higher *EGS1* transcript levels result in an increased accumulation of eugenol in different tissues.

Additionally, the *EGS1* transcript levels in leaf tissues of the ten red holy basil accessions varied greatly (Figure 4). Among the ten accessions, OS01 had the highest *EGS1* transcript levels, while OS08 showed a significantly ($P < 0.05$) lower relative transcript level compared to the other accessions (Figure 4). Notably, *EGS1* is highly expressed in the leaf tissue of fragrant accessions. The first four accessions (OS01 to OS04) have aroma profiles of strong fragrance and had significantly higher levels of *EGS1* than the other less fragrant accessions (Figure 4). Eugenol is one of aroma volatiles in aromatic plants, including rose, orchid and holy basil. Its abundance is controlled by expression of *eugenol synthase* (*EGS*) genes. This gene is found high expression in various tissue for fragrance emission. In fragrant orchid, *EGS* is specific expressions in floral tissue, that is the key source for fragrance emission in *Gymnadenia* orchid and there is no expression in leaf tissue [1]. In rose, *RcEGS1* has its highest expression in the petals, the major sites of scent emission, compared to non-scent tissues. In addition, it is higher expression levels at booming state (strong fragrance) and lower levels at budding and senescence stages [14]. In our experiment, *EGS1* was higher expressed in leave, the main fragrant emission tissue of holy basil, than in flower (Figure 3). These expression levels correlated with higher eugenol content in leaves than in flowers (Table 3). These results suggest that *EGS1* transcript levels are related to tissue fragrance. Taken all together, these suggest that expression of *EGS* is associated with eugenol accumulation leading to enriched aroma in rose, orchid as well as in holy basil. Additionally, OS01 showed the highest *EGS1* expression levels among accessions, suggesting that this red holy basil accession may prove to be an excellent source for further usage in the food, cosmetic, and pharmacological industries.

In summary, our biochemical and gene identification investigation of local Thai red holy basil accessions showed genetic differences. *EGS1* transcript expression in different tissues from red holy basil accessions correlated with eugenol content. Eugenol is abundant in red holy basil, while methyl eugenol is more prevalent in white holy basil. Leaf tissues contained more bioactive constituents and higher *EGS1* gene expression than flower spikes. This information on red holy basil will support further efforts on *EGS1* gene editing to improve the functional identification of *EGS1* and eugenol biosynthesis.

4 Conclusions

The bioactive constituents in Thai red holy basil differ from those of white holy basil, especially within the phenylpropanoid group that includes eugenol and methyl eugenol. Eugenol of Thai red holy basil was highly detected in among 2.25% and 3.79% in fresh and dry leaves tissues, respectively. Whereas methyl eugenol was the highest (3.71%) detected in fresh leaves of white holy basil. Thai red holy basil *EGS1* is closely related to white holy basil *O. tenuiflorum* and lemon basil *O. americanum*. The detection of *EGS1* transcript expression in leaf tissue was greater than 1.24-fold changed than that of spike flower tissue. Among ten accessions of red holy basil, *EGS1* expressions were strongly expressed in OS01, OS04, OS02, and OS03, respectively. OS01-OS04 were determined to be the best candidates of Thai red holy basil for further studies on the biological activities incorporated into its beneficial applications. This study emphasizes the importance of genetic diversity among Thai red holy basil accessions and provides valuable reference plants for *EGS1* gene information and secondary metabolites.

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Author Contributions

S.S.: study conception and design, investigation, writing, reviewing, and editing; W.S.: investigation and data

analysis for GC-MS; P.M.: Investigation and data analysis for gene expression; N.L.: research design, data analysis; S.I.: research design and revision of the manuscript; C.M.: study conception and design, revision of the manuscript; All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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