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Molecular characterization of tocopherol biosynthesis genes from *Olea europaea* (L.) cv. Ayvalık

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Abstract: Vitamin E is a group of compounds that includes metabolites known as tocopherols, which have high antioxidant activities. Tocopherols are synthesized in plants, and their beneficial effects on human health have been reported for diseases such as coronary heart disease and cancer. Here we report the full-length transcripts encoding vitamin E biosynthetic enzymes from fruit mesocarp tissues using the rapid amplification of cDNA ends (RACE) method for the first time in olives. We characterized the structure of the genes 4-hydroxyphenylpyruvate dioxygenase (*PDS1*), homogentisate phytyltransferase 1 (*HPT1*), vitamin E defective 3 (*VTE3*), tocopherol cyclase (*VTE1*), and gamma tocopherol methyltransferase (*GTMT*), which are responsible for tocopherol biosynthesis in the olive cultivar Ayvalık. Although *PDS1* is widespread in all organisms, *HPT1*, *VTE3*, *VTE1*, and *GTMT* are only present in photosynthetic organisms. We isolated total RNA from the dissected mesocarp tissues of fruit collected at 15-day intervals between October and December 2014. We compared the expression levels of genes using quantitative RT-PCR and determined the tocopherol content using the high-performance liquid chromatography–fluorescence detection (HPLC-FLD) technique during fruit maturation in the Ayvalık cultivar. High *OeHPT1*, *OeVTE3*, *OeVTE1*, and *OeGTMT* expression was noted in young fruit. However, the *OePDS1* mRNA did not exhibit significant expression changes during maturation. The α-tocopherol content varied between 26.78 and 21.05 mg/100 g and was highest at the early stages of fruit development. In addition, expression studies and tocopherol content revealed that tocopherol biosynthesis in olive is more active at the early stages of fruit maturation.

Key words: *Olea europaea*, tocopherol, vitamin E biosynthesis, gene expression

1. Introduction

Antioxidants scavenge reactive oxygen species to protect cells from their cytotoxic effects (Fryer, 1992). Cells produce reactive oxygen species as a by-product of several metabolic pathways and increase their production in response to biotic or abiotic stress. When accumulated in cells, reactive oxygen species can damage DNA, protein, and lipids (Gechev et al., 2003; Hurst et al., 2004). Thus, antioxidants, such as tocopherols, maintain the balance between production and scavenging of reactive oxygen species.

Vitamin E is a group of compounds including tocopherols, metabolites with high antioxidant effects (Kamal-Eldin and Appelqvist, 1996). Tocopherols are mostly synthesized by oil plants and are known for their beneficial effects on human diseases such as coronary heart disease and cancer (Rimm et al., 1993; Yang et al., 2012). In plants, tocopherols are involved in plant responses to abiotic stress, cell membrane stability, signal transduction, and control of light stress in chloroplasts (Munné-Bosch and Alegre, 2002). In addition to their antioxidant effects, tocopherols have other functions, including prevention of lipid oxidation (Frankel, 1996), cell signaling, and gene regulation (Azzi et al., 2004; Rimbach et al., 2010).

Five proteins, including methyltransferase and cyclase enzymes, participate in tocopherol biosynthesis (Lushchak and Semchuk, 2012). The PDS1 enzyme initiates biosynthesis via 4-4-hydroxyphenyl pyruvic acid (p-HPPA) and produces homogentisate (Norris, 1998). Then HPT1 synthesizes 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) from homogentisate (Collakova, 2003). MPBQ is the precursor of all tocopherol forms. VTE3 and VTE1 synthesize γ-tocopherol from MPBQ (Porfirova et al., 2002). GTMT transfers a methyl group to γ-tocopherol and forms α-tocopherol (Koch et al., 2003).

Genes involved in tocopherol biosynthesis were previously characterized in different model organisms (DellaPenna and Pogson, 2006; Quadrana et al., 2013), and recent studies revealed an association between tocopherol content and expression levels of genes

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involved in tocopherol biosynthesis (Georgiadou et al., 2015). In this paper, we present full-length cloning and bioinformatic analysis of five olive genes involved in tocopherol biosynthesis for the first time. We also performed expression analysis and determined tocopherol content for five sampling dates for the Ayvalık cultivar and discussed our results with other cultivars from previous studies.

2. Materials and methods

2.1. Plant material

We collected fruit from *Olea europaea* cv. Ayvalık trees growing in the Edremit Olive Production, Training and Gene Center at 15-day intervals on five sampling dates (between 7 October and 2 December 2014). Fruit samples were immediately frozen in liquid nitrogen and stored at –80 °C until RNA isolation. One fruit sample from 3 different trees was taken as a biological group for qPCR. In total, 100 olives from 3 different trees were collected on each sampling date for α-tocopherol analysis.

2.2. RNA isolation

Total RNA was extracted from mesocarp tissues from three fruit samples (1 sample from 3 different trees) for all sampling dates and used in rapid amplification of cDNA ends (RACE) and qRT-PCR studies. Mesocarp tissue was ground in liquid nitrogen and RNA isolation was performed with TRIzol (Ambion, 15596026) and an RNA mini kit (Ambion, 12183018A) according to the manufacturer's instructions. RNA integrity was assessed by electrophoresis, and the concentration was measured with a Qubit fluorometer (Invitrogen, Q32866). RNA was treated with DNase I (Thermo Scientific, EN0523).

2.3. Characterization of transcripts

We downloaded the short reads of the olive transcriptome data in fastQ format from the NCBI sequence read archive (SRA) and obtained a local library. Then we blasted *Arabidopsis thaliana* sequences as a query against this library and assembled the obtained reads with low E-values. We trimmed the low-quality reads using Geneious R8 software (Kearse et al., 2012). We used these short reads (~300–400 bp) to design primers for RACE reactions with Geneious R8 software (Kearse et al., 2012) (Supplementary Table). Then 3' and 5' RACE procedures were performed as previously reported (Scotto-Lavino et al., 2006, 2007). We used P-F, H-F, V3-R, V1-R, and G-F primer pairs for 3' RACE amplification and P-N-F, H-N-F, V3-N-R, V1-N-R, and G-N-F for nested 3' amplification. P-R, H-R, V3-F, V1-F, and G-R primer pairs were used for cDNA synthesis in 5' RACE. P-N-R, H-N-R, V3-N-F, V1-N-F, and G-N-R primer pairs were used to amplify 5' RACE products, and P-E-R, H- E-R, V3-E-F, V1-E-F, and G-E-R primer pairs were used for nested 5' RACE PCR. PCR products were gel purified with a PureLink Quick Gel Extraction Kit (Thermo Fisher Scientific, K2100) and sequenced. Then these sequences were used to design primers for qPCR and amplify the full-length transcript. Primers used to amplify full-length transcripts were G-FL-F and G-FL-R for *GTMT*, H-F-F and H-FL-R for *HPT1*, P-FL-F and P-FL-R for *PDS1*, V3-FL-F and V3-FL-R for *VTE3*, and V1-FL-F and V1-FL-R for *VTE1*. Full-length transcripts were amplified with Q5 High-Fidelity DNA Polymerase (New England Biolabs-M0491S), isolated from gels, and subjected to sequencing.

2.4. Bioinformatic analysis

We used Geneious R8 (Kearse et al., 2012) software to predict amino acid sequences. Molecular weight and isoelectric point of proteins were predicted using Expasy's ProtParam server (http://web.expasy.org/protparam/) (Gasteiger et al., 2005). Subcellular localization was predicted by CELLO version 2.5, a subcellular localization predictor (http://cello.life.nctu.edu.tw/) (Yu et al., 2006). Amino acid sequences were analyzed for conserved regions with the Conserved Domain Database (CDD) (Marchler-Bauer et al., 2015). To analyze 5' upstream promoter regions of tocopherol biosynthetic genes, we used a recently sequenced genome assembly of olive Farga cultivar (Cruz et. al., 2016). We used a BLAST search to identify genomic regions and retrieved –1000 bp upstream sequences. The PlantCARE Database was used to analyze motifs found in promoter regions (Lescot, 2002).

Amino acid sequences of homologues were obtained from the NCBI protein database. Sequences were aligned using default parameters with Clustal Omega software (Sievers et al., 2011). Phylogenetic trees were constructed using Mega 6 software by the neighbor joining method with the Poisson model and 1000 bootstrap replications (Tamura et al., 2013).

2.5. Expression study

Expression levels of tocopherol biosynthesis genes were analyzed by Step One Real Time PCR system (Applied Biosystems, 4376357) as per the manufacturer's instructions. The efficiency of primers for SYBR Green-based qPCR was calculated with standard curve experiments. *GAPDH2* was used as an endogenous control, as it was reported to be suitable for qPCR experiments with olive fruit samples (Nonis et al., 2012; Resetic et al., 2013). Primers used for qPCR experiments included V3-E-F and V3-E-R for *VTE3*, V1-E-F and V1-E-R for *VTE1*, H-E-F and H-E-R for *HPT1*, G-E-F and G-E-R for *GTMT*, and P-E-F and P-E-R for *PDS1*. First-strand cDNA synthesis was performed after DNase I treatment with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) using 1 µg of total RNA according to the manufacturer's instructions. Three biological and 3 technical replicates were used, and the

data were analyzed with Data Assist Software version 3.0.1 (Applied Biosystems). Controls lacking RT or template were included on each plate to assess the PCR reaction for genomic DNA contamination. Reactions were prepared with 300 nM primers, 1 µL of cDNA of 50 ng/µL, and 5 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, 4367660). Cycling conditions were 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and 1 min at 72 °C. One-way ANOVA (Field, 2007) and Tukey's test (Tukey, 1951) was used to analyze statistically significant changes at $P < 0.05$.

2.6. Determination of tocopherol content

We collected 100 olives for each sampling date and calculated their maturity index (Morello et al., 2004). In total, 50 g of mesocarp tissue was homogenized and centrifuged at 9000 rpm for 15 min. The upper oil phase was separated and stored at –20 °C until needed. Then α-tocopherol content was analyzed with HPLC-FLD at the Scientific and Technological Research Council of Turkey (TÜBİTAK) Marmara Research Center, Food Analysis Labs.

3. Results

3.1. Tocopherol biosynthesis genes in olive

Tocopherol biosynthesis was studied in several species and has been reviewed (Chaudhary and Khurana, 2009). Five genes were characterized as encoding tocopherol biosynthesis enzymes (Dellapenna and Pogson, 2006) (Figure 1). To identify these genes in olive, we used olive transcriptome data retrieved from the NCBI website (SRP000653). Although these data were obtained from various genotypes, including Carotina and Tendellone, we were able to align the raw sequences after trimming low-quality reads using Geneious R8 software. The alignment allowed us to design primer pairs using conserved regions between various genotypes. We used the RACE technique to sequence these transcripts, analyzed their structure via a bioinformatic approach, and measured expression levels in ripening fruit mesocarp.

3.2. Structure of tocopherol biosynthesis genes

We obtained partial mRNAs for all genes from the olive SRA transcriptome library and used this sequence information to design primer pairs for RACE studies. First, we amplified the 3' and 5' ends. To obtain the full-length cDNA, we designed a new set of primers and sequenced a transcript and deposited it in GenBank. A BLAST search of the transcript and amino acid sequences against tocopherol biosynthesis genes of *Arabidopsis thaliana* and *Solanum* spp. showed high similarity (Table 1).

We predicted the coding regions and analyzed the amino acid sequences for protein characteristics, predicted localizations, and conserved domains (Table 2). The results revealed similar conserved regions and localizations for all genes. Thus, we designated these sequences as *OePDS1*, *OeHPT1*, *OeVTE3*, *OeVTE1*, and *OeGTMT*.

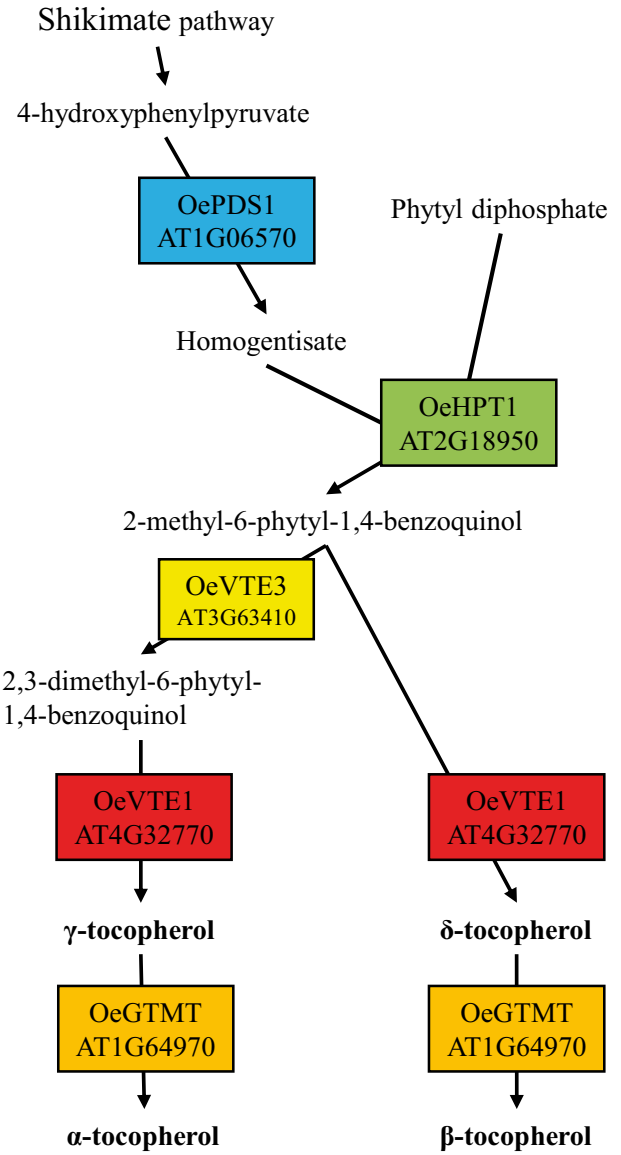

Figure 1. Pathway and genes of tocopherol biosynthesis in *Arabidopsis thaliana* and *Olea europaea*.

3.3. Analysis of promoter regions

To identify transcriptional regulatory elements in the promoter regions of tocopherol biosynthesis genes, 5' –1000 bp upstream regions were analyzed. To better visualize the results between all genes, we generated a heat map indicating the presence or absence of a motif (Figure 2). The core TATA box and CAAT box promoter elements were identified in all genes. Similarly, all genes contained Skn-1 and box 4 motifs. The Skn-1 motif is a cis-acting regulatory element required for endosperm expression, and box 4 is involved in light responsiveness (Blackwell,

Table 1. Tocopherol biosynthesis genes in olive and their similarities to model plant species.

Similarity of olive protein sequence to *Solanum* spp.*	Similarity to *Arabidopsis thaliana* protein sequence	Similarity to *Arabidopsis thaliana* sequence	Arabidopsis Locus ID	Accession number	Length of the transcript	Gene name
80%	74%	68%	AT1G06570	KU727768	1524	*OePDS1*
84%	78%	75%	AT2G18950	KU727769	1285	*OeHPT1*
83%	76%	76%	AT3G63410	KU727770	1231	*OeVTE3*
73%	68%	74%	AT4G32770	KU727771	1624	*OeVTE1*
80%	74%	68%	AT1G64970	KT777643	1298	*OeGTMT*

*Sequences used included *S. lycopersicum PDS1* (ABE41795), *S. lycopersicum HPT1* (XP_004242907.1), *S. lycopersicum VTE3* (XP_004247204.1), *S. lycopersicum VTE1* (XP_004245276.1), and *S. tuberosum* GTMT (ABE41795).

Table 2. Characteristics of olive tocopherol biosynthesis proteins.

Gene name	Protein length	Molecular weight (Da)	Isoelectric point	Predicted localization	Domains
OePDS1	450	49282.6	6.03	Cytoplasm, chloroplasts	N and C terminal HPPD_Like domains
OeHPT1	297	33190.6	9.44	Plasma membrane	Tocopherol phytyltransferase domain
OeVTE3	330	37127.9	9.20	Chloroplast	S-adenosylmethionine-dependent methyltransferases (SAM, AdoMet-MTase)
OeVTE1	515	58124.7	6.35	Cytoplasm, chloroplast	tocopherol cyclase domain
OeGTMT	327	36109.1	5.76	Chloroplasts	S-adenosylmethionine-dependent methyltransferase (SAM, AdoMet-MTase)

1994). A total of 19 motif regions associated with light response were identified with different distributions in all genes. An ethylene responsive element was identified in all genes, with the exception of Oe*PDS1*. An ABRE, which is involved in abscisic acid responsiveness (Hobo 1999), was identified in Oe*PDS1*, Oe*HPT1*, and Oe*VTE3*. Three MYB binding sites involved in light response and flavonoid biosynthesis were identified with different distributions on all genes (Figure 2).

3.4. Phylogenetic analysis of tocopherol biosynthesis genes

We conducted a phylogenetic analysis of tocopherol biosynthesis genes for a data set consisting of homologues from various organisms, including angiosperms and animals (Figure 3). All genes were widespread across the plant kingdom, and monocot and dicot plant species were separated in different clades. All tocopherol biosynthesis genes were grouped in the dicot clade with *Sesamum indicum*. As the first enzyme in the pathway, the PDS1 tree included a *Homo sapiens* protein in addition to plant species. Hydroxyphenyl dioxygenase (PDS1) proteins are present in animals, bacteria, and plants. These proteins play roles in tyrosine catabolism and contribute to tocopherol biosynthesis in plants (Gunsior et al., 2004). The *PDS1* clade also included *Chlamydomonas reinhardtii*, as tocopherol biosynthesis is also present in algae (Sattler et al., 2003; Backasch et al., 2005). The *HPT1* clade included monocot, dicot, algae, and cyanobacteria groups. OeHPT1 was grouped with other dicot plants. The VTE3 clade also included *Chlamydomonas reinhardtii*, which is an outgroup to monocot and dicot species.

3.5. Expression levels of tocopherol biosynthesis genes on fruit mesocarp

To identify expression patterns of tocopherol biosynthesis genes during fruit maturation, we analyzed five different samples and found that all genes are expressed. The maturity indexes of fruit samples ranged between 2.07 (more than half of the fruit is green) to 4.08 (purple fruit at harvest maturity). No tocopherol biosynthesis genes exhibited drastic changes (>2-fold); however, increased expression was found at early sampling dates. Oe*PDS1* expression analysis through olive fruit maturation did not reveal any statistically significant changes with one-way ANOVA (Field, 2007) and Tukey's test (Tukey, 1951) at $P < 0.05$. During the early steps of olive maturation (7 October 2014 and 21 October 2014 samples), Oe*HPT1* exhibited

Motifs	Function	PDS1	HPT1	VTE3	VTE1	GTMT
3-AF1 binding site	light responsive element					
5UTR Py-rich stretch	high transcription levels					
AAGAA-motif	light responsiveness					
ABRE	abscisic acid responsiveness					
ACE	light responsiveness					
AC-II	regulation of phloem expression					
AE-box	part of a module for light response					
ARE	essential for the anaerobic induction					
as-2-box	light responsiveness					
AT1-motif	part of a light responsive module					
ATCT-motif	light responsiveness					
Box 4	light responsiveness					
box E	fungal elicitation					
Box I	light responsive element					
Box-W1	fungal elicitor responsive element					
CAAT-box	common element in promoter and enhancer regions					
CAT-box	meristem expression					
CGTCA-motif	MeJA-responsiveness					
circadian	circadian control					
ERE	ethylene-responsive element					
GAG-motif	part of a light responsive element					
GA-motif	part of a light responsive element					
GARE-motif	gibberellin-responsive element					
GATA-motif	part of a light responsive element					
GC-motif	anoxic specific inducibility					
HSE	heat stress responsiveness					
I-box	part of a light responsive element					
LTR	low-temperature responsiveness					
MBS	flavonoid biosynthetic genes regulation					
MRE	light responsiveness					
MSA-like	cell cycle regulation					
O2-site	zein metabolism regulation					
OBP-1 site	cis-acting regulatory element					
P-box	gibberellin-responsive element					
Skn-1_motif	endosperm expression					
Sp1	light responsive element					
TATA-box	core promoter element					
TATCCAT/C-motif	sugar repression responsiveness					
TCA-element	salicylic acid responsiveness					
TGA-element	auxin-responsive element					
W box	wounding and pathogen responsiveness					

Figure 2. Heat map of motifs found in promoter regions of tocopherol biosynthesis genes. Red indicates presence and white indicates absence of a motif.

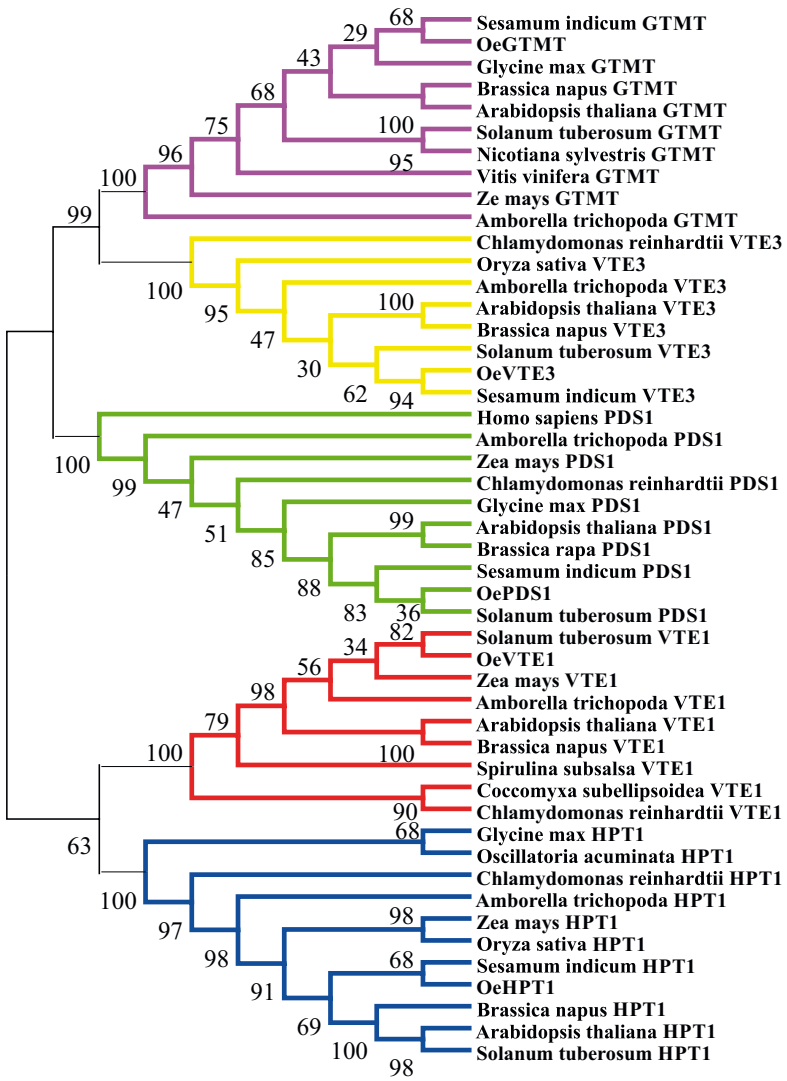

Figure 3. Phylogenetic tree of tocopherol biosynthesis genes of olive and model species constructed using the neighbor joining method with the Poisson model. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are indicated near the branches.

increased expression levels relative to earlier sampling dates. *OeVTE3*, *OeVTE1*, and *OeGTMT* exhibited increased expression levels in the 7 October 2014 samples compared with earlier sampling dates (Figure 4).

3.6. Tocopherol content of the Ayvalık cultivar

The maturity indexes of fruit samples were 2.07 for 21 October, 3.0 for 4 November, 3.50 for 18 November, and 4.08 for 2 December. These maturity indexes indicate that our samples included fruit coloring and harvest maturity stages. The tocopherol content of Ayvalık cultivar oil samples ranged from 26.78 to 21.05 mg/100 g, with the highest levels noted in the 21 October samples (Figure 5). The later samples did not exhibit major changes through fruit maturation.

4. Discussion

We sequenced and characterized 5 olive transcripts that exhibit high similarities to plant tocopherol biosynthesis genes. We suggest that these transcripts encode PDS1, HPT1, VTE3, VTE1, and GTMT enzymes in olive due to sequence similarity, predicted subcellular localization, and conserved domains. Tocopherol biosynthesis genes are widely spread across the plant kingdom. In plants, *PDS1* is involved in tocopherol and plastoquinol-9 biosynthesis. In animals, *PDS1* is involved in tyrosine degradation (Moran, 2005). Consistent with tocopherol biosynthesis being a plant-specific process, the *HPT1*, *VTE3*, *VTE1*, and *GTMT* genes were exclusively identified in photosynthetic organisms. In silico analysis of subcellular localization revealed that olive

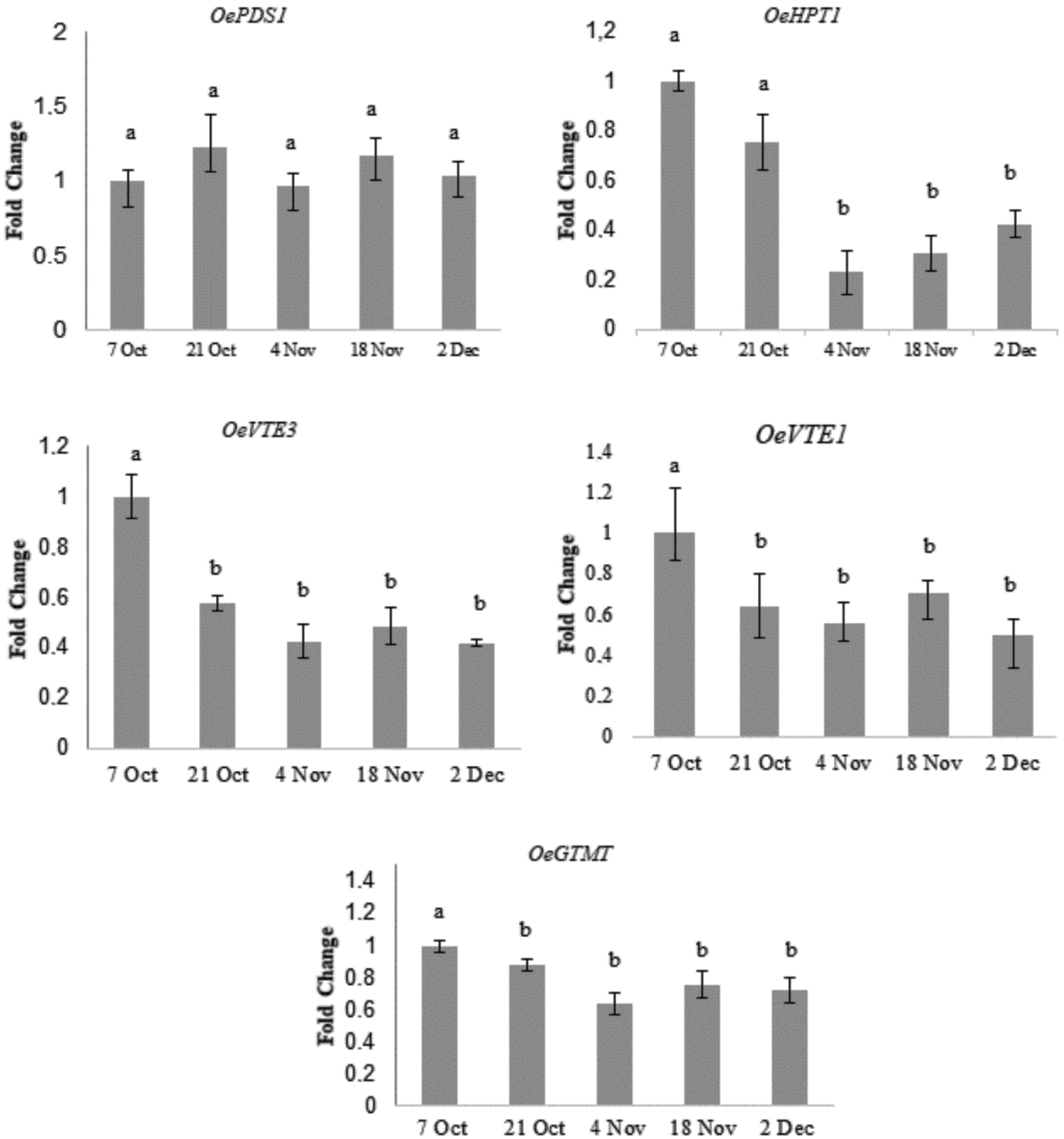

Figure 4. Relative expression levels of tocopherol biosynthesis genes during fruit ripening between 7 October 2014 and 2 December 2014. Error bars represent standard deviation of the mean. Different letters represent significantly different groups of one-way ANOVA (Field, 2007) and Tukey's test (Tukey, 1951) at $P < 0.05$.

tocopherol biosynthesis genes were mostly localized in both the chloroplasts and cytoplasm. Although CELLO predicted that Oe*HPT1* and *A. thaliana HPT1* were localized in the plasma membrane, previous reports revealed HPT1 localization in the chloroplast membrane (Yang et al., 2011). CELLO did not make such a prediction for OeHPT1. The promoter regions of tocopherol biosynthetic genes included core promoter elements, such as the TATA box and CAAT box; 19 different motif regions involved in light responses; and regions involved in plant hormone responses (e.g., ethylene, abscisic acid, auxin, gibberellin), heat stress, low temperature responses, and pathogen responses.

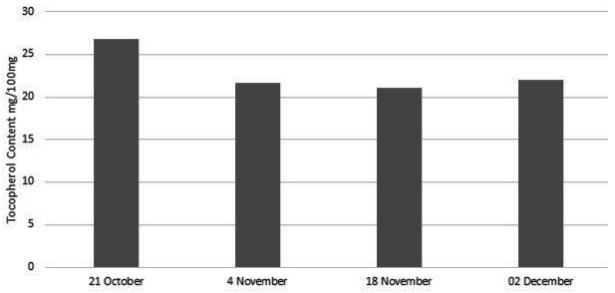

Figure 5. Tocopherol content of *Olea europaea* (cv Ayvalık) oil extracted from mesocarp tissues.

Light response regions were widely common in all genes. Tocopherols are involved in light stress (Munné-Bosch and Alegre, 2002, Munné-Bosch, 2005). In *Arabidopsis*, excess light conditions affect α-tocopherol levels and the expression profiles of tocopherol biosynthesis genes (Ksas et al., 2015). Thus, light-responsive regions in olive tocopherol biosynthesis genes could be related to the response to light stress conditions. In addition, *OeHPT1*, *OeVTE3*, *OeVTE1*, and *OeGTMT* each contained an ethylene-responsive element, which may control tocopherol biosynthesis via the role of ethylene role in fruit ripening. Although it is known that ethylene levels increase throughout the fruit ripening process (Lelievre et al., 1997), tocopherol levels exhibit different patterns in different olive cultivars. Although Hojiblanca (Beltran et al., 2005) and Koroneiki (Georgiadou et al., 2015) exhibited an increase, Verdial and Villalonga (Garcia et al., 1996) exhibited a decrease in α-tocopherol content. In addition, the arbequina and lechin (Garcia et al., 1996) cultivars did not exhibit significant changes. Moreover, various olive cultivars growing in Turkey exhibited genotype-specific α-tocopherol content (Şeker et al., 2008). These results suggest that α-tocopherol content in olive fruits might be affected by ethylene, but other factors more likely affect the process.

The expression levels of tocopherol biosynthesis genes did not exhibit drastic changes throughout fruit maturation. Although *OeVTE3*, *OeVTE1*, and *OeGTMT* expression was increased in the 7 October samples among the five sample groups, we did not identify any statistically significant change in later samples (21 October, 4 November, 18 November, 2 December). A recent study revealed expression levels of tocopherol biosynthesis genes and tocopherol content in the Koroneiki olive cultivar (Georgiadou et al., 2015). The authors found that the expression levels of *GTMT* were increased before fruit coloring but reduced during later stages, such as fruit coloring and harvest maturity. They observed an upregulation of *VTE3* and *VTE1* during early developmental stages but a downregulation during later stages (Georgiadou et al., 2015). These results are consistent with the Ayvalık cultivar, given that we also observed downregulation of *OeVTE3*, *OeVTE1*, and *OeGTMT* at the late maturation stages. *OeHPT1* showed upregulation at 7 October and 21 October compared with samples collected at later stages (4 November, 18 November, and 2 December 2014). The Koroneiki cultivar also exhibited reduced *HPT1* expression levels at later stages (Georgiadou et al., 2015). The authors found that *PDS1* expression peaked 30 weeks after flowering and was downregulated drastically at later stages. Our results did not exhibit significant expression changes within the 5 samples for *OePDS1*. Our results showed that the α-tocopherol content of the Ayvalık cultivar was between 26.78 and 21.05 mg/100 g, with the highest levels noted for the 21 October samples. The results from the Koroneiki cultivar revealed similar values within the same stages of maturation (Georgiadou et al., 2015). Both cultivars exhibited increased α-tocopherol content at early stages of maturation. These data show that differential expression of *PDS1* between the Koroneiki and Ayvalık cultivars may not affect tocopherol content in a cultivar-specific manner. Therefore, we suggest that *PDS1* is not a limiting factor for vitamin E biosynthesis in olive. *PDS1* and *VTE3* also have roles in plastoquinol-9 biosynthesis, and their expression could be associated with this metabolic process. The expression levels of other genes related to this pathway did not exhibit variation between different genotypes. Several factors could alter tocopherol expression and α-tocopherol accumulation, such as abiotic stress and developmental change of fruit (Munné-Bosch, 2005; Abbasi et al., 2007; Miret and Munné-Bosch, 2015). The expression data and tocopherol contents suggest that tocopherol synthesis starts at early stages of fruit maturation. Although we did not analyze other tocopherol forms, such as β-tocopherol, γ-tocopherol, or tocotrienols, their analysis could provide a better understanding of *VTE1* and *VTE*3 expression. Additionally, in our work, we did not analyze the enzymatic activities that may also be related to tocopherol content. Olive genotypes show cultivar-specific tocopherol content patterns that might affect oil quality content (Şeker et al., 2008). However, the enzyme activity of tocopherol biosynthesis proteins and the antioxidant activity of tocopherols in olive fruits and oil could also affect the quality of oils from olive genotypes. Further functional studies and in vitro studies could reveal the relationship between tocopherol biosynthesis gene expression and abiotic stress. These results will provide valuable data on olive breeding strategies like selecting and improving olive genotypes with high vitamin E content.

Acknowledgments

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Supplementary Table. Primer sequences used for qPCR and RACE.

Gene	Primer name	Primer sequence (5'– 3')	Procedure used
OePDS1	P_F	TTCCGCTACCACGGGGTTTA	3' RACE
OePDS1	P_R	GGGCAGTGAAGAGGAATTGGA	5' RACE
OePDS1	P_N_F	AACTGGTGGGGTACAACAACTT	3' RACE
OePDS1	P_N_R	AAGATAGGAGGCGTGGACGG	5' RACE
OeHPT1	H_F	TGCATTGAGTCCAAGATGCAG	3' RACE
OeHPT1	H_R	CTCCCGATGCCAATGGAAGA	5' RACE
OeHPT1	H_N_F	TCTAGTGAGTGCAACTTCTGAAC	3' RACE
OeHPT1	H_N_R	AGACAGCTGATTCAAACCGACT	5' RACE
OeVTE3	V3_F	CCCATGTCTACGAACCCCAC	5' RACE
OeVTE3	V3_R	AGGCTAAGAAGAAGGAGCCC	3' RACE
OeVTE3	V3_N_F	TTTTGGACCAATCCTCTTCAGTTG	5' RACE
OeVTE3	V3_N_R	ATGCTGAGGACCTCCCTTTCA	3' RACE
OeVTE1	V1_F	AGCATCAGTTCATTCCTACTCCC	5' RACE
OeVTE1	V1_R	CCCTAATGTTCAGAAGCCGGT	3' RACE
OeVTE1	V1_N_F	TCTGAGATTCTTCAGTGTATTGGCA	5' RACE
OeVTE1	V1_N_R	GATGTTACGGAGGCTCCCAG	3' RACE
OeGTMT	G_F	CAGGACAAATAGTTGATGTGGGT	3' RACE
OeGTMT	G_R	CACTCTCCATGGACCAAACCA	5' RACE
OeGTMT	G_N_F	AGGTAGTTCAAGGTACTTGGCG	3' RACE
OeGTMT	G_N_R	TGCCCATCCTGAAATGGTTGATT	5' RACE
OeGTMT	G_FL_F	ACTCCTTTTCCAGCCATGC	Full length amplification
OeGTMT	G_ FL _R	AATTGTTCAACATTCAATTACAGGA	Full length amplification
OeHPT1	H_ FL _F	CAAAAGCATCTCCGCACTAA	Full length amplification
OeHPT1	H_ FL _R	AAGATATTGTGTACAATCACAAATGC	Full length amplification
OePDS1	P_ FL _F	CACCGCTTCCGCTACCAC	Full length amplification
OePDS1	P_ FL _R	TTTGCATCAATTTACCTATCTTGG	Full length amplification
OeVTE3	V3_ FL _F	AACCACGAGTACTTTCCATCTACTT	Full length amplification
OeVTE3	V3_ FL _R	TTCAATGAGCAAAAACAGGTACCATA	Full length amplification
OeVTE1	V1_ FL _F	CCCACCAGTCCTCTCTACCC	Full length amplification
OeVTE1	V1_ FL _R	TGCAATATTTACACAACAATATAAACA	Full length amplification
OeVTE3	V3_E_F	TGGACCAATCCTCTTCAGTTGA	Expression studies
OeVTE3	V3_ E_R	GCTGATGTGTGGATGCTCTTC	Expression studies
OeVTE1	V1_ E_F	GCCTCCTCTAGTCTGTTCAATCT	Expression studies
OeVTE1	V1_ E_R	GCTGGTACTTCAAGGTGTCAATAC	Expression studies
OeHPT1	H_ E_F	ACACAATCTTGAATCCGAACCTTC	Expression studies
OeHPT1	H_ E_R	TGCTCAATGCTGTTCCTATTACTG	Expression studies
OeGTMT	G_ E_F	GGTTGTGGTATAGGAGGTAGTTCA	Expression studies
OeGTMT	G_ E_R	TTGAGCAGCAGCAAGAGC	Expression studies
OePDS1	P_ E_F	CGATGCCACCAACACCTC	Expression studies
OePDS1	P_ E_R	GGAGATTTACAGAGCGGAGAAGA	Expression studies