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Analysis of *OeMVK* Gene Expression in Different Olive Tissues Using Real Time PCR

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Abstract

Olive tree (*Olea europaea* L.) that is a member of the Oleaceae family, is an evergreen, small tree that have been cultivated since prehistoric times in the eastern Mediterranean region. Olive is one of the vital components of Mediterranean diet. The pharmacological properties of olive oil, the olive fruit, and its leaves have been recognized as important components of a healthy diet as well as medicine because of their active role in diseases management. Mevalonate Kinase (MVK; EC 2.7.1.36; ATP:(R)-mevalonate 5-phosphotransferase) is the first enzyme in the plant isoprenoid biosynthesis in MVA (Mevalonate) pathway. In this study the *MVK* (Mevalonate Kinase) gene was cloned successfully from *Olea europea*, and named *OeMVK* (accession number: MH427085). The ORF (Open Reading Frame) of *OeMVK* was 1164 bp. OeMVK protein was consisted of 387 amino acids. Homologous sequence analysis showed that amino acid sequence of OeMVK had the highest identity of 97% with *Catharanthus roseus* mevalonate kinase 2a. Real-time PCR assay demonstrated that *OeMVK* was constitutively expressed in all tissues of olive with a similar transcription level with a slightly increase in unripe fruit. The molecular characterization of olive MVK gene and its expression level were speculated by taking the all olive tissues into account.

Keywords: *Olea europea*, Olive, Gene expression, Mevalonate kinase

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Farklı Zeytin Dokularında *OeMVK* Gen Ekspresyonunun Gerçek Zamanlı PZR Kullanarak Analizi

Öz

Oleaceae familyası üyesi zeytin ağacı (*Olea europaea* L.), doğu Akdeniz bölgesinde tarih öncesi zamanlardan beri kültüre alınmış, her daim yeşil ve küçük bir ağaçtır. Zeytin, Akdeniz diyetinin en önemli unsurlarından biridir. Zeytinyağının, zeytin meyvesinin ve yapraklarının farmakolojik özellikleri, hastalık yönetimindeki aktif rolleri nedeniyle, sağlıklı bir diyetin yanı sıra ilacın da önemli bileşenleri olarak kabul edilmiştir. Mevalonat Kinaz, (MVK; EC 2.7.1.36; ATP:(R)- mevalonat 5-fosfotransferaz) bitkilerde MVA (Mevalonat) yolağında isoprenoid biyosentezinin birinci enzimidir. Bu çalışmada *Olea europea*' ya ait MVK (Mevalonat Kinaz) geni başarı ile klonlanmış ve *OeMVK* (erişim numarası: MH427085) olarak isimlendirilmiştir. *OeMVK* genine ait ORF (Open Reading Frame) 1164 bp uzunluğundadır. OeMVK protein 387 amino asitten oluşmaktadır. Homolog dizi analizlerine göre OeMVK proteinini en yüksek benzerliği %97 ile *Catharanthus roseus* mevalonat kinaz 2a ile göstermiştir. Anlık gösterimli PZR ile OeMVK' nın zeytine ait tüm dokularda üretildiği ve transkripsiyon seviyesinin ham meyvede bir miktar daha fazla olmakla birlikte tüm dokularda aşağı yukarı benzer bir seviyede olduğu gözlenmiştir. Zeytin MVK geninin moleküler karakterizasyonu ve transkripsiyon seviyesinin tespiti için tüm zeytin dokuları ile çalışılmıştır.

Anahtar Kelimeler: Olea europea, Zeytin, Gen ekspresyonu, Mevalonate kinaz

1. Introduction

Mevalonate (MVK; EC 2.7.1.36; ATP:(R)- mevalonate 5-phosphotransferase) is an early enzyme in plant isoprenoid biosynthesis. In the plant, specific mevalonate kinase activities were found to be relatively high in the fruits, stem, roots, flowers and buds, and relatively low in young and completely elongated leaves [1]. Three highly conserved motifs characterize MVKs. Motif I: contains partial active site (PGKVILXGEHSVVXXXPAz); motif II: a glycine-rich motif (SIGXGLGSSAG) that creates a phosphate-binding loop in all GHMP kinases, and motif III: a conserved motif (KLTGAGGGGC) that stabilizes the phosphate binding loop [2]. Peroxisomal targeting signals (PTS1 and PTS2) were also analysed in *Arabidopsis thaliana* for the presence of

consensus signals for MVA pathway enzymes and their similarity to the consensus sequences of their respective human MVA pathway enzymes. The consensus sequence "KIILAGEHA" was detected as PTS2 motif 1 in *Arabidopsis thaliana* at similar positions relative to their respective N terminus [3].

Isoprenoid biosynthesis is one of the major physiologically important pathways in plants leading to greater array of compounds such as abscisic acid, chlorophyll, ubiquinone, sterols and phytoalexins which play important roles in the growth and development of the plant [4-6]. The common precursor of all isoprenoids is isopentenyl diphosphate (IPP) and there are two pathways that are known in the biosynthesis of IPP, pathway'' or ''MEP pathway'' that occurs in the plastids [7-9].

Studies on the MVK gene are limited. The previous studies about MVK gene were mostly about gene cloning, transcriptome analysis and MVK activity. The corresponding MVK gene has been cloned from *Catharanthus roseus* [1], *Arabidopsis thaliana* [10], *Salvia miltiorrhiza* [11], *Panax notoginseng* [12], *Zea mays* [13], *Chamaemelum nobile* [14], *Hevea brasiliensis* [15] and so on. There are also some studies indicating that MVK might play a role in the regulation of isoprenoid biosynthesis. [1, 10, 16].

Olive (*Olea europaea* L., family Oleaceae) and known mostly as the olive tree, is an evergreen tree from 12 to 20 ft high, silver-green leaves, with hoary, rigid branches and a grayish bark [17]. The olive tree was one of the earliest fruit crops to be domesticated both for oil and fruit production [17, 18]. Olive is one of the emblematic crops of the Mediterranean region, where most of the world's olive oil is produced Olive (*Olea europea* L.). Olive oil is a highly priced product, due to the difficulties concerning the cultivation, harvest and the limited production in the areas [19].

Olive (*Olea europaea* L.) fruits contain numerous secondary metabolites, primarily phenolics, terpenes and sterols, some of which are particularly interesting for their nutraceutical properties. The presence of many mono and sesquiterpenes, even in low amounts in the unripe fruits must be pointed out as these compounds disappears in ripe olives [16, 20]. In many countries, extract of *Olea europaea* is used in the treatment of diarrhea, dysentery, fever, diabetes, and hypertension [21].

Previous studies about olive MVK were limited and only about the intermediate compounds formed by the reactions catalysed by this enzyme and real time PCR experiment by the EST sequence to determine the expression level of the enzyme on the 3 level of fruit formation (45, 90, 165 days after flowering) that did not contain any information about molecular and biochemical characterization olive MVK gene and enzyme [16]. Being for the first time of the molecular characterization of this gene in olive is the original value of this study.

This article reports the isolation, molecular characterization, and tissue-specific expression analysis of OeMVK (MK gene of *Olea europaea* L.) as a first step in understanding on the early steps of isoprenoid biosynthesis pathway in olive. The results of this study are expected to shed penetrating insights into the regulation of secondary metabolite biosynthetic pathways by ubiquitous enzyme OeMVK in an important medicinal plant, olive.

2. Materials and Methods

2.1. Sample Collection, RNA Isolation and cDNA Synthesis

Olive samples were collected from Adıyaman Directorate of Provincial Food Agriculture and Livestock. See Table 1 for the detailed sampling. The collected tissues were immediately transferred in liquid nitrogen and stored in -80 °C freezer until usage. Total RNA isolation was performed using the RNeasy Plant Mini Kit (Qiagen, Germany) with on-column DNAseI digestion from Olive (*Olea europaea* L. cv. Ayvalik) samples. cDNA was synthesized using total RNA with a RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to the manufacturer's instructions. To reveal the open reading frame of *OeMVK*, degenerate primers were designed by using Primer 3 programme [22] according to the sequence showing highest homology, *Catharanthus roseus* mevalonate kinase 2a (HM462019.1), mevalonate kinase mRNA sequence (GenBank accession no. HM462019.1). PCR was performed using Quick-Load® *Taq* 2X Master Mix (NEB, U.K.) with forward (5'- AAGGAAATGGAGGTAAGAGCTAGAG -3') and reverse (5'- ATGAAAAACCAGTGAAGGAAATCT - 3') primers according to the manufacturer's protocol. PCR products were sequenced by a biotechnology company (SENTEGEN, Ankara).

Table 1: Sampling olive tissues throughout the year

Olive Tissue	Months											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Leaf	X	X	X	X	X	X	X	X	X	X	X	X
Unripe fruit							X	X	X	X		
Ripe fruit	X	X									X	X
Bud				X								
Flower					X							
Pedicel					X							

2.2. Protein Sequence and Phylogenetic Analysis

The protein sequence was analysed with various bioinformatics tools, namely ExPASy [23], I-TASSER [24] and BIOEDIT [25], to explore the OeMVK protein. The ExPASy proteomic server [23], was used to analyse physicochemical property of the protein based on primary sequence of OeMVK. The 3D structure of the protein was calculated by I-TASSER [24], and full-length cDNA sequence of *OeMVK* was revealed via PCR using cDNA from olive leaves as a template. The primers used in full length sequence determination and sequencing were mentioned in Materials and Methods section 2.1. Primary sequence of OeMVK protein was identified by using BIOEDIT [25] and subjected to Basic Local Alignment Search Tool (BLAST) search against non-redundant (NR) database of National Centre for Biotechnology Information (NCBI). The sequences showing significant homology (identity cut-off, >75%; query coverage, >80%; and E value $\leqslant$0) were aligned using ClustalW tool [25]. The phylogenetic tree was constructed using the set of aligned sequences by implementing the neighbour-joining method in PAUP 4.0b10 [26]. A bootstrap replication of 1000 was used as it is preferable for estimating the reliability of the phylogenetic tree.

2.3. Expression profile determination by real-time PCR

Primers used in this study were selected according to the previous study [16]. 1 µL of 10 µM each forward (5'- TGGCAAATGTTTGAGGATAGTG -3') and reverse (5'- GCGTGTCAATTCACCAGACTTA -3') gene specific primers (5 µM each), cDNA and sterile H_2O were added to the lyophilized FastStart Essential DNA Green Master (Roche, Switzerland) to set up the real-time PCR reactions which were run on an LightCycler® 96 System (Roche, Switzerland). The thermocycler was first heated to 95 °C for 3 min. This was followed by a step of 94 °C for 5 seconds and 35 cycles of 94 °C for 25 seconds

/ 55°C for 25 seconds and 72 °C for 30 seconds. GAPDH was proved to be an effective normalizer gene for olive and hence was utilized in this work as well [27-29]. Each PCR was run in triplicates and the significance of the differences were determined through statistical analyses (see below).

2.4. Statistical Analysis

All measurements were run in triplicates and the results were considered reliable when the standard error values (calculated with the spreadsheet's respective function) were less than 10%. For the statistical analysis, analysis of variance (ANOVA) was performed by using Minitab 17 Statistical Software [30]. For the differences, $p<0.05$ was considered significant, and the significance was indicated with an asterisk (*) on the plots.

3. Results and Discussion

3.1. Isolation and Bioinformatic Analysis of *OeMVK*

In this study, the gene *OeMVK* encoding MVK of the MVA pathway and the enzyme catalysing the first step in this pathway, were characterized. MVK gene was previously studied in other plants, but there is limited information about the molecular characterization of the gene [14-15]. The nucleotide sequence was designated as *OeMVK* and GenBank accession number was KX894317. *OeMVK* gene from *Olea europaea* cv. Ayvalik (Gen Bank accession no: MH427085) consisted of an open reading frame of 1164 bp.

Although the BLAST analysis showed that the cDNA sequence is highly similar to the other plant MVK sequences, especially to *Catharanthus roseus* mevalonate kinase with 97% similarity (HM462019.1), *OeMVK* showed over 98% similarity to the sequences of the other varieties of *Olea europea* L., that have been studied and registered to the GenBank (Accession number; (XM_023010363.1), (XM_023010362.1), (JX266177.1), (JX266176.1) and (XM_023014264.1)). (Fig. 1).

3.2. Characterization of OeMVK protein

The deduced protein was consisted of 387 amino acids. This protein had a 41 kDa mass and its theoretical pI was 5.46 according to the online bioinformatic tools of

ExPASy [23], and Bioedit biological sequence alignment editor [25]. The predicted 3D structure of the protein and the surface map was obtained by the I-Tasser software [26]. Elements of secondary structure are coloured by rainbow N → C terminus and contain alpha helix (40%), beta strand (21%). OeMVK 3D model showed similar fashion in other MVK proteins (Fig. 2a) [2, 3]. A conserved glycine-rich sequence "GSGLGSSA" which forms a phosphate-binding loop in all GHMP kinases, a conserved amino acid sequence "KLTGAGGGGC" that stabilizes the phosphate binding loop and a conserved motif contains "KIILAGEHA" PTS2 motif which is consisted of a part of the active site at similar positions as in Arabidopsis thaliana Mevalonate Kinase (NM_180753) and other plant MVK proteins [3].

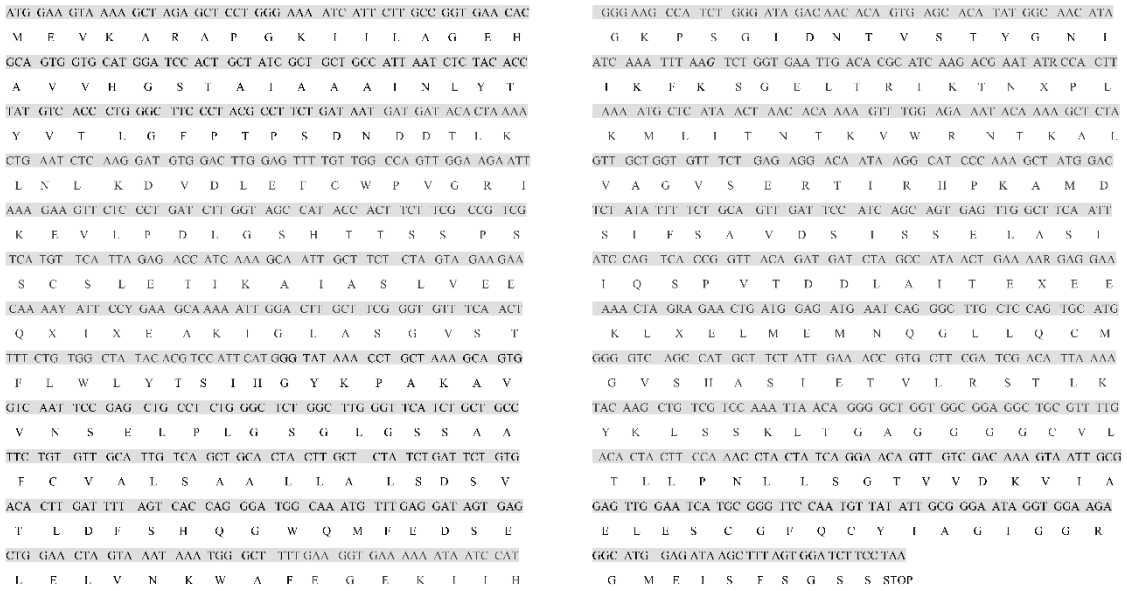

Figure 1. The ORF (Open Reading Frame) and amino acid composition of *OeMVK* gene. The cDNA is shown in grey highlight and the amino acids are shown below

Bioinformatics analysis revealed that the deduced OeMVK harboured a highly similar identity to MVKs of other plants. (Fig. 2b) [10, 14]. The deduced amino acid sequences of a phylogenetic tree of mevalonate kinases (amino acid sequences) from closely related plants also revealed that OeMVK was closest to *Catharanthus roseus* mevalonate kinase (HM462019.1) (Fig. 2c).

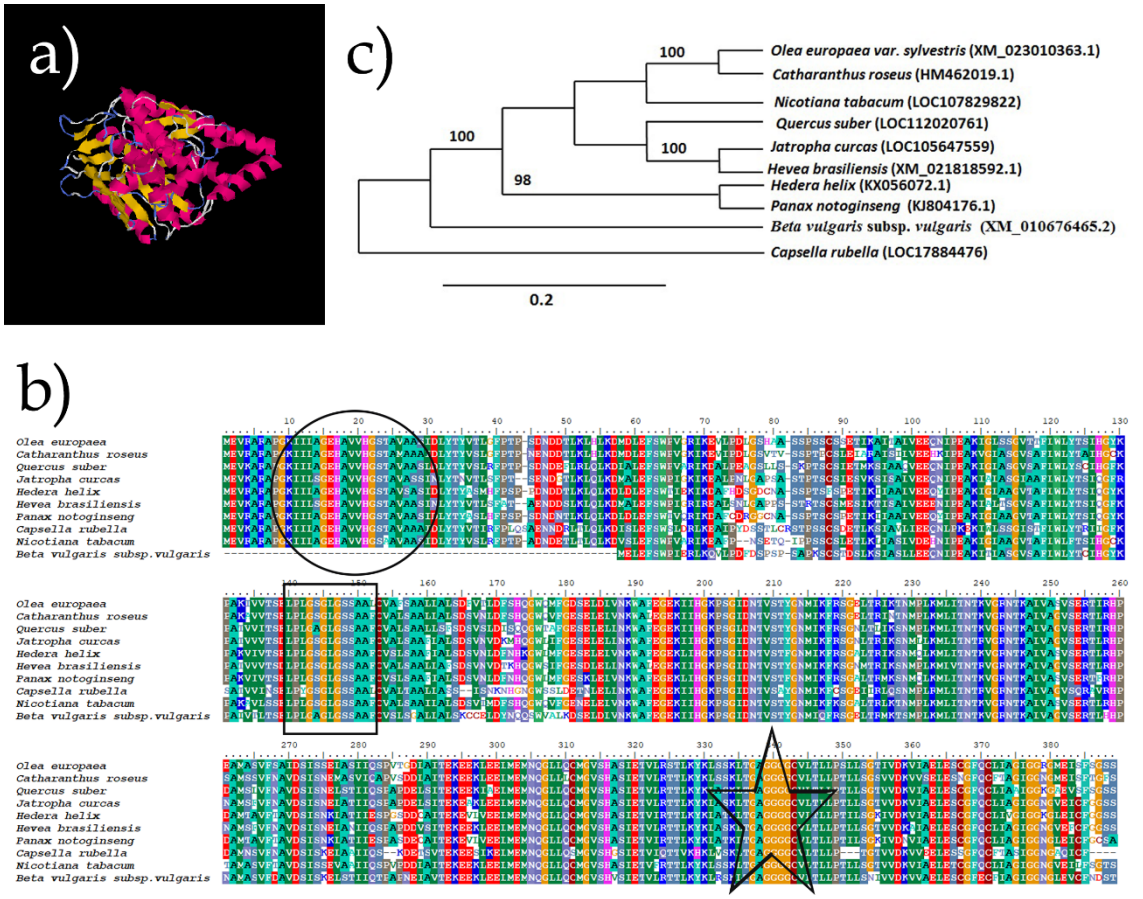

Figure 2. Characterisation of OeMVK protein using bioinformatics tools. a) 3D structure of predicted OeMVK protein by I-Tasser software. Those coloured ones are the residues of OeMVK in the β-sheet folding domain and the α-helix domain. b) Multiple sequence alignment of MVK proteins, the completely identical amino acids are indicated with the same colour. The conserved motifs; motif 1: "KIILAGEHA", motif 2: "GSGLGSSA" and motif 3: "KLTGAGGGGC" among plant MVK proteins was shown in circle, rectangular and star, respectively. c) Polymorphism among different plant mevalonate kinase proteins

3.3. Tissue Expression Pattern Analysis of *OeMVK*

Total RNA was isolated from different organs, including roots, stems, leaves, fruits, and flowers of *O. europaea* and a detailed temporal and spatial expression pattern of *OeMVK* was determined using real time subjected to RT-PCR. The GAPDH gene expression in all the detected tissues were used as internal control PCR. cDNA templates were used from various olive tissues and leaves throughout the year (Fig. 3). The results suggested that OeMVK expression was more or less the same in the leaves that were collected from the same trees every month throughout the year (Fig. 3a). The result showed that OeMVK expression could be detected in all organs at different levels. In all the olive tissues examined, the data suggested that OeMVK was mostly expressed in early stages of fruit (Fig. 3b).

In a previous study dealing with the metabolic and transcriptional profiling during fruit development in *Dolce d'Andria* and *Coratina* varieties also confirmed that OeMVK was mostly expressed in the first developmental stage of the fruit and there are some differences of the expression levels between olive varieties [20], which could be explained by altered accumulation of isoprenoids during the fruit development.

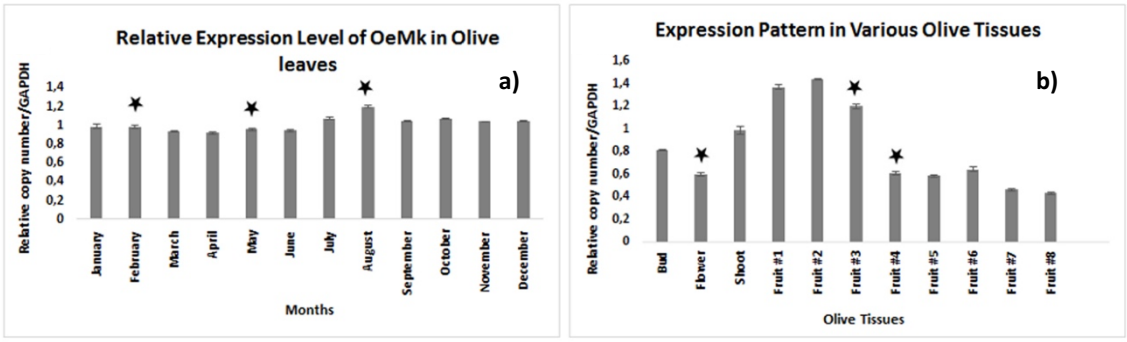

Figure 3. Real-time PCR results displaying mRNA expression level. *OeMVK* expression levels from leaves a) and from different tissues, b) The values displayed are the means ± SE (n=3). One asterisk (✶): $p<0.05$ compared to the corresponding value by ANOVA that was calculated using the Minitab 17 software [26]

4. Conclusion

In this study, *OeMVK* gene was successfully isolated from *Olea europaea*. The ORF of the gene is 1164 bp encoding 387 amino acids. The multiple sequences alignment showed that *OeMVK* had high similarity with other plant MVK genes. The phylogenetic analysis indicated that OeMVK was strongly conserved while keeping the diversity among plant species. This study provides data for the molecular characterisation of *OeMVK* and can contribute to the further studies dealing with the regulation of terpenoid biosynthesis.

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