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The molecular cloning and structural analysis of a cytochrome P450 (*CYP71D500*) encoding gene from ajowan (*Trachyspermum ammi* L.) medicinal plant

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ABSTRACT

Plant phenolic monoterpenes, such as thymol and carvacrol, have a wide range of applications in medicinal, pharmaceutical and other industries. Ajowan is an aromatic medicinal plant from the *Apiaceae* family with thymol as an active component of its seeds. The seeds of ajowan are valuable for medicinal purposes because their essential oil contains active substances of thymol, carvacrol, γ-terpinene, and p-cymene. Cytochrome P450 (CYP) –related genes have an indispensable role in the biosynthetic pathway of thymol and other substances in ajowan. A cytochrome P450 gene (*CYP71D500*) with a high expression level was isolated from the ajowan, cloned and sequenced. The sequencing information from the previous RNA-Seq study confirmed that the isolated gene belongs to the plant CYP71 clan, with a 1654 bp length containing two exons and a 115 bp intron. The full-length cDNA of *CYP71D500* was also cloned. The sequencing of *CYP71D500* cDNA showed the complete homology of *CYP71D500* cDNA and exon regions of the *CYP71D500* genomic sequence. The sequencing analysis of *CYP71D500* cDNA also revealed a mutation that changed isoleucine to valine amino acid. The 3D structural analysis of the enzyme showed that the modified amino acid is not located in the enzyme's active site. Therefore, this cannot probably affect the synthesis of thymol. The isolated gene and cDNA could be used for the metabolic engineering of ajowan and other medicinal plants with active phenolic monoterpenes. It is also applicable for identifying the different functions of the cloned *CYP* gene in ajowan or other medicinal plants.

Key words: Ajowan, Apiaceae, Cytochrome P450, CYP71D500, Metabolic engineering, Monoterpenes.

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INTRODUCTION

Medicinal plants are valuable green factories supplying many drug requirements by producing secondary metabolites (Noori *et al.,* 2020; Guo *et al*., 2022). In addition to their role in protecting plants against different biotic and abiotic stresses, plant secondary metabolites are valuable natural products with plenty of applications in various industries (Amiri *et al.*, 2020). Plant-derived medicines have specific popularity among consumers and different societies because of their organic nature with fewer side effects (Niazian *et al.*, 2017). Ajowan is an important medicinal plant from the *Apiaceae* family with many useful applications in traditional and advanced medicine. The seeds of ajowan are rich in volatile aromatic compounds like thymol, carvacrol, γ-terpinene, and p-cymene which have contributed to its medicinal properties (Boskabady *et al.*, 2014; Mirzahosseini *et al.*, 2017; Soltani Howyzeh *et al.*, 2018a). Thymol and carvacrol as the two important phenolic monoterpenes with antiherbivore, antimicrobial, pharmaceutical and antioxidant properties, can be found in high amounts in this medicinal plant (Soltani Howyzeh *et al.*, 2018c; Krause *et al.*, 2021).

Thymol, as the most valuable pharma molecule in ajowan, is a monoterpene and phenol derivative of cymene (Sadat-Noori *et al.*, 2018). Bioactive terpenoids are valuable natural products due to their nutrients, flavours, fragrances, insecticidal and herbicidal properties, and their medicinal role (Pateraki *et al.*, 2015). The terpenoid backbone formation is the intermediate step in the biosynthesis of monoterpenoids in ajowan. The terpene synthases, under the control of a large number of putative terpene synthase (*TPS*) unigenes, are involved in the formation of cyclic monoterpenes from geranyl diphosphate (GPP). However, the initially produced monoterpenes are usually oxidized or conjugated by the cytochrome P450s (CYP) enzymes (Crocoll, 2011; Soltani Howyzeh *et al.*, 2018b; Amiripour *et al.*, 2019). The major enzymes of the cytochrome P450s have an important role in the functionalization of the core terpene molecules (Pateraki *et al.*, 2015; Calla and Berenbaum, 2020). Therefore, discovering and analysing plant *CYP450* genes have an important role in identifying terpenoid biosynthetic pathways. Exploring the genes involved in the pathway of medicinal compounds especially cytochrome genes is the most dynamic field of medicinal plant genetic research (Di and Gilardi, 2020; Wang *et al*., 2021). In addition to producing plant pharma molecules, CYP450s have other functional roles in their carrier plants. Balusamy *et al.* (2019) isolated cytochrome P450 monooxygenase (*CYP736B*) cDNA from *Panax ginseng* and transferred it to the *Arabidopsis* model plant. They reported that overexpression of *PgCYP736B* in *Arabidopsis* increased resistance to salt stress. In addition, to enhance tolerance to the abiotic stress, because of high sequence homology with *CYP736A12*, *PgCYP736B* may also contribute to triterpenoid biosynthesis in ginseng (Balusamy *et al.*, 2019). Therefore, manipulating *CYP450* genes in medicinal plants can, directly and indirectly, affect their secondary metabolite production.

The endogenous presence of all required components, such as isoprene precursors, cofactors, and critical enzymes, make plants perfect platforms for the biotechnological production of terpenoids (Pateraki *et al.*, 2015). Various biotechnology-based breeding methods (BBBMs) can accelerate the improvement of medicinal plants. The metabolic engineering (ME) of medicinal plants is one of these BBBMs that can be used for the overproduction of desired pharma molecules of medicinal plants through overexpression of the genes involved in the key biosynthesis pathways (Niazian, 2019). Metabolic engineering is an effective strategy to enhance thymol content in ajowan and other medicinal plants. Although some efforts have been conducted to increase the thymol content of ajowan using biotechnology and genetic engineering (Niazian *et al.*, 2019; Nomani *et al.*, 2019), none of them used biosynthesis regulation of endogenous genes. The manipulation of ajowan thymol content through the terpenoid backbone pathway is a difficult task because the key enzymes of this pathway are controlled by multiple copies of *TPS* unigenes with different expression patterns in different plant genotypes.

In addition, the role of cytochrome P450 enzymes in thymol formation is obvious (Amaeze *et al.*, 2021). The metabolic engineering (ME) of ajowan through regulation of cytochrome P450s endogens could be a promising strategy to increase its thymol and other valuable secondary metabolites. However, identification, isolation, and cloning of candidate genes are prerequisites for the overproduction of useful natural products by ME. The previous transcriptome profiling (RNA-seq) studies showed the significant effect of unigenes of the *CYP450* gene family in the biosynthesis of monoterpenoids in ajowan (Amiripoor *et al.*, 2018; Soltani Howyzeh *et al.*, 2018b; Amiripour *et al.*, 2019). The aim of the present study was to (i) clone and sequence *the CYP71D500* gene and related cDNAs occurring in the ajowan, and (ii) analyse the structure of its coding sequence. The cloned gene and

Table 1. Forward and reverse primer sequences for the amplification of some ajowan genes and pTZ57R/T cloning vector.

Gene name	Forward (5'-3')	Reverse (5'-3')
CYP71D500	ATGCATTATACACCAATGGAATTCC	TTAGTTAACAACTTCAATTGGGTATTC
SAND	TTGTAAGCTGAGTCTGTAATCCATC	CCTAAAGTGACCAGAAACACAAG
eIF-4a	CATGCGTGAGTTCCGTTCTG	GCAGCTCCTCGACAACAACCAC
actin	GTGTTATGTCTTGGCGTCTTG	TATGCAGACCATGTCAGTAGAG
M13 (vector)	GTAAAACGACGGCCAG	CAGGAAACATCGATGACC

the corresponding cDNAs based on previous research studies (Amiripoor *et al.*, 2018; Soltani Howyzeh *et al.*, 2018b; Amiripour *et al.*, 2019) can probably be used to increase the thymol content in ajowan and other medicinal plants from the *Apiaceae* family and for functional analysis of the *CYP71D500* gene.

MATERIALS AND METHODS

Plant materials

The seeds of Arak ecotype of ajowan were provided by the department of agronomy and plant breeding science at the College of Aburaihan-University of Tehran. Seeds were cultivated in plastic pots (10 cm in diameter and 15 cm in height) and grown in the greenhouse (in temperature 25 °C with 60% relative humidity under 16/8 [light/dark] photoperiod) of the National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran. Regular irrigation was conducted at two days intervals until the emergence of plantlets and then irrigation was conducted with five days intervals. The morphological changes of inflorescences were assessed (Soltani Howyzeh *et al.*, 2018a), and the late flowering stage (5 days after anthesis), was selected as the best developmental stage for RNA extraction. The highest expression level of *CYP* genes has been previously reported in this period (Soltani Howyzeh *et al*., 2018b).

DNA and RNA extraction

DNA was extracted from selected leaf samples according to the protocol developed by Sika *et al.* (2015). RNA was extracted from leaf tissues using TRIZOL reagent (Cat#15596026, Invitrogen, USA) according to the manufacturer's instructions. Agarose gel electrophoresis was used to assess the quality of extracted DNA and RNA. The quantity of extracted DNA and RNA was evaluated by NanoDrop (Thermo Scientific™, USA).

PCR amplification and electrophoresis recovery of *CYP71D500* gene

Primers of *the CYP71D500* gene were designed using mRNA information of *CYP* genes from a previous RNA-Seq study on ajowan (Soltani Howyzeh *et al.*, 2018b). Very high homology for the beginning and end of the gene was determined, and forward and reverse-specific primers of *the CYP71D500* gene were designed using Primer3 software (Table 1). *The* temperature gradient method (annealing temperature between 53-60 °C) was conducted to find the best annealing temperature in the PCR test. The amplification was performed using a thermal cycler (MyCycler, BioRad, USA) programmed as follows: initial denaturation at 95 °C for 3 min, 33 cycles of denaturation (95 °C for 1 min), annealing (53-60 °C for 1 min), extension (72 °C for 1 min), and final extension at 72 °C for 10 min. The DNA polymerase enzyme was ExPrime Taq DNA Polymerase (High Fidelity) from GeNet Bio (Daejeon, South Korea). PCR products were separated on a 1% (w/v) agarose gel. Then, ethidium bromide visualized bands were recovered using the Roche High Pure PCR Product Purification Kit (Cat# 11 732 668 001, Germany) according to the manufacturer's instructions. PCR analysis was carried out for three proposed housekeeping genes of ajowan, including *SAND*, *eIF-4a*, and *actin* genes (Soltani Howyzeh *et al.*, 2018b) as well. The primers for these genes are presented in Table 1.

Molecular cloning of the *CYP71D500* gene into the pTZ57R/T cloning vector

The TA cloning reaction (Thermo Scientific Fisher) was conducted to clone the isolated *CYP71D500* gene into the pTZ57R/T cloning vector. The reaction components, including pTZ57R/T vector, isolated *CYP71D500* gene (recovered PCR product), ligation buffer, and T4 DNA Ligase were mixed together and kept at 22 °C for at least one hour. The ligation mixture was then transferred to DH5α strain of *E. coli* competent cells using the heat shock method (Froger and Hall, 2007). The accuracy of transformation was assessed using the colony-PCR test and digestion pattern analysis (Skorupski *et al*., 1996).

Sequencing of cloned *CYP71D500* gene

The recombinant pTZ57R/T cloning vector, harboring ajown *CYP71D500* gene, was extracted

from transformed cells of the DH5α strain using Plasmid DNA Isolation Kit (Cat# K-1000, GeNet Bio, Korea). The sequencing of the cloned *CYP71D500* gene was conducted using M13 forward and reverse primers (Table 1). The alignment of the obtained sequence with the information from the NCBI database was performed using MegAlign software (DNASTAR).

Reverse transcription-PCR (RT-PCR) and cloning of *CYPD71D500* cDNA

The extracted RNA (DNase treated) was used to synthesize cDNA using Thermo Fisher Scientific RevertAid First Strand cDNA Synthesis Kit (Cat# K1622, USA). The PCR program was conducted as follows: initial denaturation at 95 °C for 10 sec, 33 cycles of denaturation (95 °C for 1 min) - annealing (60 °C for 1 min) - extension (72 °C for 90 sec), and final extension at 72 °C for 10 min. The RT-PCR product was extracted and recovered from 1% (w/v) agarose gel and then cloned into the pTZ57R/T cloning vector (Thermo Scientific Fisher) by the TA cloning method (Palatinszky *et al.*, 2011). The recombinant vector was transferred to the DH5α strain of *E.coli* using the heat shock method (Zhou and Gomez-Sanchez, 2000). The positive transformed colonies were selected on LB-agar plates containing ampicillin and then checked by the colony-PCR method and digestion pattern analysis.

Sequencing *CYP71D500* cDNA and determination of the functional effect of gene mutations

The Universal M13 forward and reverse primers were used for sequencing the cloned *CYP71D500* cDNA (Table 1). The alignment of the obtained sequence with the information from the NCBI database was conducted using MegAlign software (DNASTAR). The spatial three-dimensional (3D) structures of the CYP71D enzyme were created using I-TASSER software. The active site of the enzyme and the location of a putative-modified amino acid(s) in the enzyme structure were predicted using Molegro Virtual Docker software.

RESULTS AND DISCUSSION

PCR -amplification of *CYP71D500* gene

The electrophoresis of PCR products on 1% (w/v) agarose gel showed the expected band of *CYP71D500* gene (1654 bp) at an annealing temperature of 60 °C (Figure 1). These results showed the efficiency of the designed primers for PCR amplification of the *CYP71D500* gene. The results of the gradient PCR of housekeeping genes also showed their expected band (about 250 bp) in the annealing temperature of 60 °C (Figure 2). Based on the electrophoresis analysis of

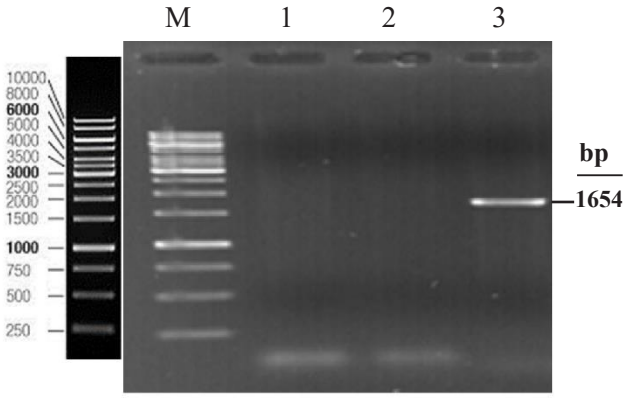

Figure 1. The PCR amplification of the CYP71D500 using designed primers. M: GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific), 1: amplified DNA fragment with expected size 1654 bp.

the PCR product, the *eIF-4a* gene showed the highest expression level between the three housekeeping genes (Figure 2). These results are in line with the previous research of the qRT-PCR reaction of these housekeeping genes on ajowan (Soltani Howyzeh *et al*., 2018b).

Molecular verification of cloning of *CYP71D500* gene into the pTZ57R/T cloning vector

The PCR-amplified *CYP71D500* gene fragment was first recovered from 1% (w/v) agarose gel and then cloned into the pTZ57R/T cloning vector using the TA cloning technique (Zhou *et al*., 2000). The competent cells of the DH5α strain of *E. coli* were then transformed by the recombinant pTZ57R/T cloning vector. Colonies containing pTZ57R/T construct were cultured on the LB-agar medium supplemented with 50 mg/L ampicillin. The colony PCR results showed the successful cloning of the *CYP71D500* gene into the pTZ57R/T cloning vector and successful transformation (Figure 3).

Sequencing of cloned *CYP71D500* gene

The comparison of the obtained sequence of the isolated gene showed a high degree of sequence homology with the mRNA sequence information present in the NCBI database. It showed that the cloned gene belongs to the *CYP71D* gene family. The complete sequence was submitted by ID: MH638297 in the NCBI's Genebank (https://www.ncbi.nlm.nih.gov/nuccore/MH638297.1/). The plant CYP71 category is the most rapidly evolving P450 family (Pateraki *et al.*, 2015) and covers more than half of all CYPs in higher plants (Nelson and Werck-Reichhart, 2011). The cytochrome P450 is one of the largest superfamily genes in the world. The CYP450s, with a wide range of duties,

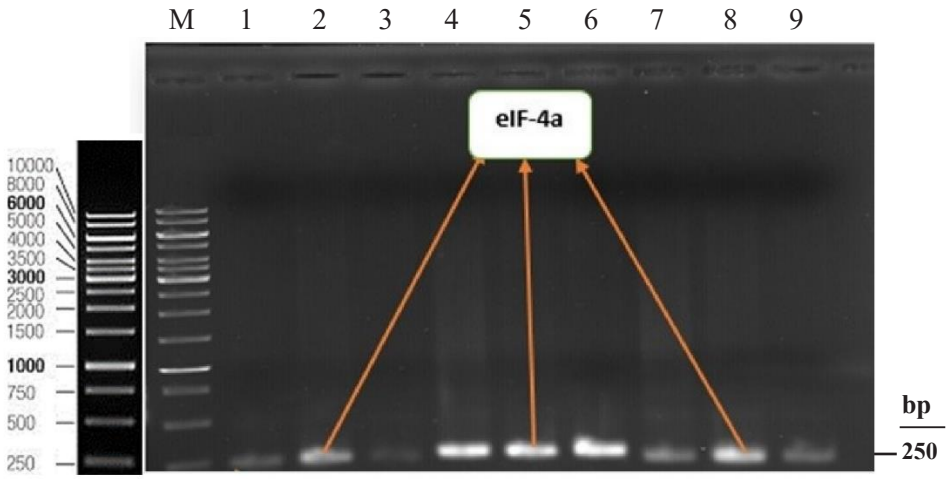

Figure 2. The PCR amplification of the housekeeping genes. M: GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific), 1-3: amplified SAND gene, 4-6: amplified eIF-4a gene, and 7-9: amplified actin gene in different annealing temperatures (expected size of all of them was 250 bp).

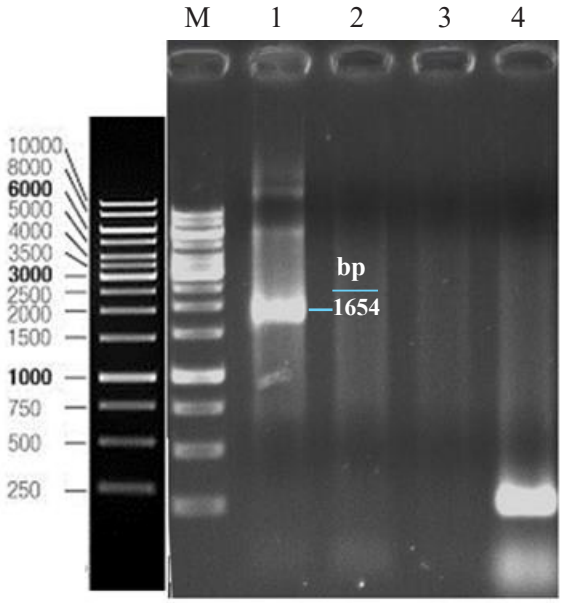

Figure 3. The colony PCR for the verification of insertion of *CYP71D500* gene into the pTZ57R/T cloning vector by m13F/R primers. M: GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific), 1: The amplified fragment of *CYP71D500* gene (expected size 1654 bp) cloned in pTZ57R/T. 2: Clone showed no amplification. 3: Negative control. 4: The amplified fragment of circular pTZ57R with the same primers.

are found in all kingdoms of life, including animals, bacteria, fungi, archaea, protists, and viruses (Crocoll, 2011; Pateraki *et al.*, 2015). These are involved in many plant biochemical pathways (Balusamy *et al.*, 2019). Metabolism of plant's important secondary metabolites, such as monoterpenoids, sesquiterpenoids, diterpenoids, and triterpenoids, are all mediated through CYP450s.

The sequencing information showed a non-similar region in the gene. The specific primers were then designed for this non-similar region and the amplified fragment was sequenced. The sequencing of this region revealed a part of two exons and a 115bp intron region in the *CYPD71D500* gene (Table 2). The existence of intron is a great advantage. Introns are important enhancers that are usually applied for the overexpression of transgenes. Different influential mechanisms have been reported through which introns can regulate gene expression (Emami *et al.*, 2013). A recent research study evaluated the effect of

Table 2. Intron sequence and its designed primers of the region located in the middle of *CYP71D500* gene of ajowan.

Gene	Sequence	Forward primer (5'-3')	Reverse primer (5'-3')
CYP71D500	GGTAAGTTTTATATTGAATTAATTCAGCTT TTTTTTTTGTTCATTTATAGTCATTACTGAT TATAACCAACTTGATGAGTACTTTTAAATC TAAAATACATGACACGGGGATGCAGGATA TGTTTACAGCCGGAACTGATACATCTT	CGTGTAGAGCCAC AATTGGAG	CCTTTCACTACCTCC CGGAT

intron region on the expression of transgene *TdSHN1* in tobacco and reported that the *TdSHN1*-intron had more expression level of the transgene and less ROS production in comparison with the intron-less construct (Djemal and Khoudi, 2019). Using the *CYPD71D500* gene, including its intron, can be helpful for ME of ajowan through overexpression of the *CYPD71D500* gene and preventing transgene silencing.

Sequencing of *CYP71D500* cDNA and structural study of coding enzyme

The *CYP71D500* cDNA band (1539 bp) was visualized after electrophoresis of RT-PCR products on 1% (w/v) agarose gel (Figure 4). Sequencing information showed a high sequence homology of the cDNA cloned in this study with *CYP71D500* mRNA sequences existing in the NCBI database. A mutation in nucleotide number 1063 changed isoleucine to valine in the translated polypeptide. The spatial and functional structures of these two amino acids are almost similar. However, the enzyme active site and the substrate insertion into the protein were assessed. The best structure of the CYP71D enzyme was selected among ten created spatial 3D models (Yang *et al.*, 2015). Then its active site and the valine amino acid position were predicted using Molegro Virtual Docker software. Results showed that valine was not located in the enzyme-active site (Figure 5); therefore, it seems this mutation has no effect on thymol synthesis in ajowan.

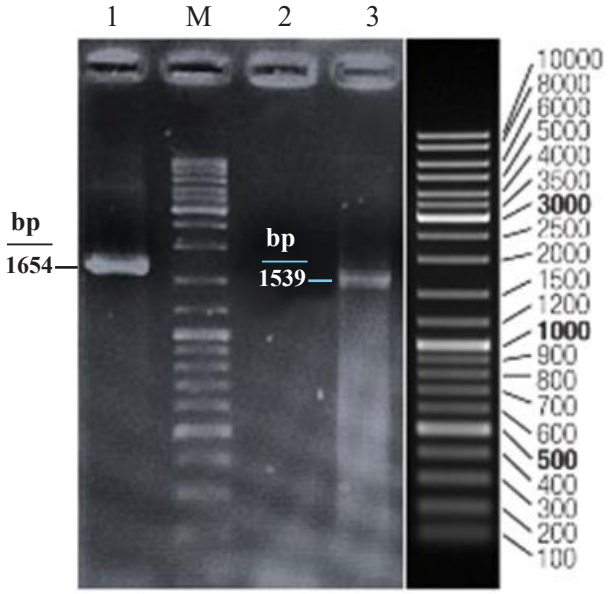

Figure 4. RT-PCR analysis of CYP71D500 cDNA. M: 100 bp DNA Ladder (Thermo Fisher Scientific), 1: The PCR amplification of intron-containing *CYP71D500* gene (expected size 1654 bp) 2: Negative control. 3: The PCR amplification of CYP71D500 cDNA (expected size 1539 bp).

CONCLUSION

Metabolic engineering is a creative strategy to increase the content of secondary metabolites in medicinal plants through the overexpression of key genes involved in their biosynthetic pathway(s) (Sethi *et al.*, 2022). Terpene synthase genes play a crucial role in the intricate biosynthetic pathway leading to the production of thymol, a well-known monoterpene compound with various pharmaceutical and aromatic applications. Understanding the genetic and biochemical processes involved in thymol biosynthesis is essential not only for the improvement of its production but also for harnessing its medicinal benefits. One of the primary players in thymol biosynthesis is the terpene synthase (TPS) gene family. TPS genes encode enzymes responsible for catalyzing the conversion of precursor molecules, such as geranyl pyrophosphate (GPP) and linalyl pyrophosphate (LPP), into diverse monoterpene compounds, including thymol. These enzymes are pivotal in determining the specific monoterpenoid products synthesized within plant cells. However, TPS genes are not the sole contributors to the complex biosynthesis of monoterpenoids like thymol. Several other gene families are also intimately involved in this process. In addition to TPS, other gene families such as cytochrome P450s (CYP450s), dehydrogenase (DHs), and transcription factors (TFs) are involved in the biosynthesis of monoterpenoids (Soltani Howyzeh *et al*., 2018b; Alicandri *et al.*, 2022; De Alvarenga *et al.*, 2023; Lanier *et al*., 2023; Williams and De Luca, 2023). Cytochrome P450s (CYP450s) are a notable

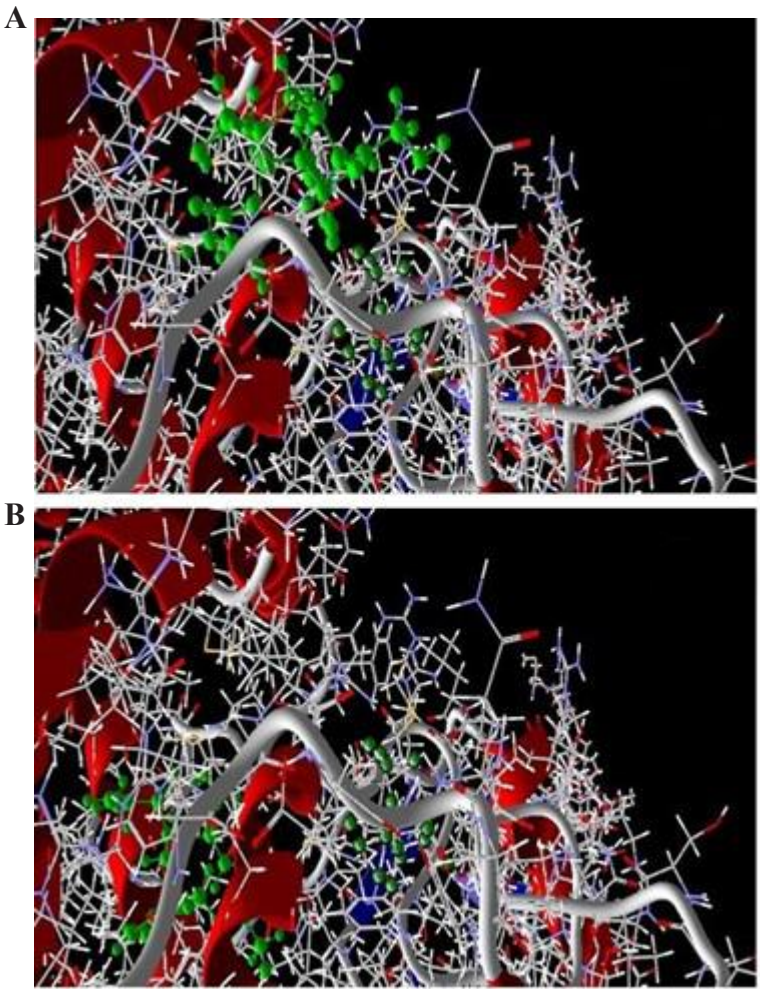

Figure 5. The spatial 3D structure of predicted CYP71D enzyme in ajowan. **A:** The positions of the active site of the enzyme in blue color and Valine amino acid as green dots outside the active side of the enzyme CYP71D. **B:** A close-up view of the active site of the enzyme and position of valine amino acid.

group of enzymes that participate in the hydroxylation and oxidation reactions of monoterpene precursors. These reactions can lead to the production of various intermediate compounds, which ultimately contribute to the biosynthesis of thymol. The interplay between TPS genes and CYP450s allows for the fine-tuning of thymol production and the generating structurally diverse monoterpenes with distinct biological activities. *TPS* and *CYP450s* have been reported as the main players in the biosynthetic pathway of terpenoid components. The collaboration among these gene families enables plants to produce thymol and other monoterpenoids. (Soltani Howyzeh *et al.*, 2018b; Trindade *et al.*, 2018). The overexpression of genes from these two well-known superfamily genes can be effective for the overproduction of thymol in ajowan. However, it seems that the manipulation of CYP450s can be more helpful because of their terminal role and ultimate impact on the post-modification of the primary terpenoid skeleton. This terminal role makes them an attractive target for metabolic engineering efforts. (Singh *et al.*, 2021). The success of a metabolic engineering program relies on a series of crucial steps. Identification, isolation, cloning, and functional and structural analysis of candidate genes are required for the schedule of an efficient metabolic engineering program. The existence of a powerful expression vector and efficient *in vitro* regeneration and gene transformation protocols are the final required items to run a scheduled metabolic engineering strategy (Sethi *et al.*, 2022). In the present study, among the three previously identified *CYP71D* genes in ajowan, the highest expressed one (*CYP71D500*) based on the previous research (Soltani Howyzeh *et al.*, 2018b) was isolated, cloned, sequenced, and inserted into the proper cloning vector. Sequencing information of the cloned *CYP71D500* revealed an intron position in its sequence, which might cause overexpression of transferred

CYP71D500. The sequencing of *CYP71D500* cDNA revealed a mutation in its sequence that changed isoleucine to valine. The 3D structural analysis of the enzyme showed that the modified amino acid is not located in the enzyme's active site. Previous researchers showed the relationship between gene mutation and the synthesis of the desired compound by that gene (Ragland *et a*l., 2014; Nawade *et al*., 2022). Therefore, this cannot affect the synthesis of thymol. Using the intron-containing gene in the final expression construct can significantly suppress gene silencing and enhance the overexpression of transgene. Gene silencing is a common challenge faced in genetic engineering, where introduced genes can be switched off or suppressed by the host plant's defenses. Incorporating introns within the gene construct is akin to including a protective shield. These introns can act as stabilizers, ensuring that the introduced gene remains active and, in fact, enhances its expression, allowing to achieve higher levels of gene production. (Vasil *et al*., 1989; Christie *et al*., 2011; Kumar *et al.*, 2022). Therefore, a cassette harboring an intron-containing gene from the CYP450 superfamily with great effect on the biosynthetic pathway of thymol can probably be very helpful for the metabolic engineering of ajowan to increase its thymol content. This research not only advances our understanding of thymol biosynthesis but also opens the door to improving the production of valuable medicinal compounds, with potential implications for various industries, including pharmaceuticals, aromatics, and agriculture.

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