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MOLECULAR CLONING AND FUNCTIONAL ANALYSIS OF FLAVANONE 3-HYDROXYLASE (*F3H*） GENE IN *PASSIFLORA EDULIS* SIMS

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Abstract

Flavanone 3-hydroxylase (F3H) plays a crucial role in the biosynthesis of flavonoids. In the present study, one *F3H* gene (P_edulia040010337.g) from *Passiflora edulis* Sims, which has a coding sequence (CDS) of 1161 bp, encoding a protein consisting of 386 amino acid residues was cloned. The PeF3H protein contains a non-heme dioxygenase (DIOX-N superfamily) domain and a typical F3H protein functional domain (2OG-FeII-Oxy dioxygenase). Phylogenetic analysis revealed that the PeF3H protein shared high similarity with F3H proteins in *Turnera subulata*, *Populus alba*, and *Populus tomentosa*, with 88% identities of amino acid sequences. The PeF3H protein lacks a transmembrane structure, indicating it is likely to be expressed in the mitochondria. Additionally, 3D structure, protein and protein interaction, and KEGG pathway of PeF3H were anticipated based on homologous proteins. qRT-PCR analysis showed that PeF3H was highly expressed in leaves, followed by stems and roots. These studies have provided insights into the molecular mechanisms underlying flavonoid biosynthesis and predicted potential targets for genetic engineering to improve the nutritional and medicinal properties of passion fruit.

Introduction

Passiflora edulis Sims, commonly known as passion fruit, is a vine plant in the Passifloraceae family. Flavonoids have excellent pharmacological effects, such as antioxidant, anti-cardiovascular disease, tumour-inhibiting, anti-bacterial, and anti-allergic effects, and are a current hotspot in natural drug research (Dias *et al*. 2021). Passion fruit is rich in flavonoids, which were often present in free or glycoside form, with the highest content found in the leaves (Zhou *et al*. 2008). Passion fruit has also been studied for its anti-anxiety, sedative, anti-bacterial, anti-inflammatory properties, and for preventing drug addiction. The leaf or flower extract from passion fruit has been used to treat symptoms such as neuralgia, insomnia, and dysmenorrhea (Pereira *et al*. 2022). However, little is known about the functional genes involved in the flavonoid biosynthesis pathway in Passiflora, which is the major pharmaceutical ingredient.

The F3H gene plays a pivotal role in the anthocyanin metabolism and accumulation pathway, as it encodes key functional enzymes that catalyze the hydroxylation of 4,5,7'-flavanone C3 to form dihydrokaempferol (DHK), a vital precursor substance for synthesizing flavanols, flavonol glycosides, and anthocyanins in plants (Hu *et al*. 2014). The F3H gene has been cloned and studied in various plants, including buckwheat, blueberry, and mulberry, etc. (Zhang *et al*. 2010, Zhang *et al*. 2017, Dai *et al*. 2022). Overexpression or inhibition of the F3H gene leads to increasing or reduction in flavonoid and anthocyanin content in plant (Wisman *et al*. 1998, Han *et al*. 2012, Li *et al*. 2014).

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The Passiflora family, the largest family of the Malpighiales, is grown commercially for its fruit, with passion fruit being cultivated worldwide and possessing great economic value (Taiwe and Kuete 2017). The recent completion of the passion fruit genome sequencing has led to the identification of the function genes at the genome-wide level, providing an opportunity to study the function of flavonoids in passion fruit further (Xia *et al*. 2021). Recently, several key gene families in passion fruit have already been studied based on the genome data (Song *et al*. 2022, Huang *et al*. 2022, Xu *et al*. 2023). In the present study, one F3H gene was cloned from passion fruit, and the biological function of the PeF3H gene was inferred through amino acid multiple sequence alignments and phylogeny tree analysis. This study insights into the structure and functional role of F3H in *P. edulis* and help identify potential targets for genetic manipulation to enhance flavonoid production in this plant.

Materials and Methods

The fresh young leaves of the purple fruit *P. edulis*, commercial name aTainong ” ainongrcial name aves of the purple fruit netic manipulation to enhance fla phylogeny tree analysis. This study insights The total RNA of leaves, stems, and roots of *P. edulis* was extracted following the instructions of the column-based Trizol RNA extraction kit. The quality of extracted RNA was checked by agarose gel electrophoresis and DeNovix ultra-micro-UV spectrophotometer. The high-quality RNA was used as a template and reverse transcript into cDNA using Thermo Fisher's reverse transcription kit and stored at -20 -20o cDNA usi

The PeF3H primers were designed using Primer 5.0 software based on the genome and transcriptomic data obtained from *P. edulis*, and the primer sequences were F3H-F: 5'-ATGCAAG GTAGGACGTTTTTTAAC-3'; F3H-R: 5'-TCAAGCTAGAATCTCTTCTGT-3'. The PCR electrophoresis gel recovery was carried out according to the column DNA gel recovery kit (Biotech kit B518131), the cloning vector pMD19-T was ligated, JM109 receptor cells were transformed, the monoclonal colonies were identified by PCR, and positive clones were selected and sequencing analysis was analysis by Shanghai Biotech Ltd.

The similarity comparison of the PeF3H gene was searched using BLAST online software; the cDNA sequence of the gene obtained by sequencing was analyzed by ORR finder of NCBI for the open reading frame; the amino acids encoded by the PeF3H gene were analyzed by Protparam (http://www.expasy.org/tools/protparam.html) for protein isoelectric point (PI), molecular weight (Mw). Jalview and MEGA11 software were used for sequence alignment and N-J phylogeny tree construction (Waterhouse *et al*. 2009, Koichiro *et al*. 2021). Phosphorylation sites of proteins were analyzed using NetPhos 3.1 Server online software (https://services.healthtech.dtu.dk /services/ NetPhos-3.1/). The secondary structures were predicted by SOPMA (http://npsa-pbil.ibcp.fr/ cgi-bin/npsa_automat.pl?page=npsa_sopma.html). The Motif Search tool (http://www.genome.jp/ tools/motif/) was used for comparative analysis of biologically significant sites of proteins. The 3D structure was predicted by SwissModel (http://swissmodel.expasy.org), blast, and AlphaFold2 (Jumper *et al*. 2021) online software, and the PDB file was downloaded and modified by PyMOL software (https://pymol.org/2/). Sub-cellular localization of proteins was performed by PSORT software (https://www.genscript.com/psort.html). The protein and protein interaction were analyzed by STRING online software (https://cn.string-db.org/). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed according to KEGG online software (https://www.kegg.jp/).

The expression of the PeF3H gene was measured in different tissues by qRT-PCR using the TransStart Tip Green qPCR SuperMix kit. The primer sequences were qF3H-F: 5'-GTGGAGGG CAGTGACAGAGG-3'; qF3H-R: 5'-GCTTGGTGGTCCGCATTCTT -3'. EF-1GAGGG5'-GTG-

GAGGG'-GTGGAGGG-F: 5'-GTGGAGGGGGGwere qF3H-F: 5'-GTGGAGGGcces were qEF-F: 5'-GGCCCAACTGG T CTGACTAC-3'; qEF-R: 5'-TTGCGGGATCATCCTTGGAG -3'. The amplification conditions were: pre-denaturation at 95 95 ration at 95 qF3H-F: 5'-GTG-GAGGGcom/psort.html). The protein and protein interaction tlysis curve was set at 72 ℃curve was set at 72 t 72 G -3'. The amplification conditions were: pre-denaturati^{-curv} method with three replicates of each reaction (Li *et al.* 2021).

Results and Discussion

The complete cDNA sequence of the F3H was isolated from the leave of P. edulis and designated as PeF3H. The open read frame of PeF3H was 1161 bp long and encoded 386 amino acid residues (Fig.1). The sequence of cloned PeF3H is fully consistent with transcriptome data.

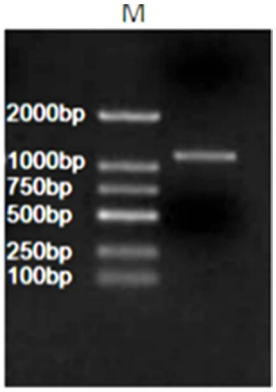

Fig. 1. PCR amplification of *PeF3H*

The deduced amino acid sequence of the *PeF3H* was alignment with the other 9 plants' F3H. The multiple alignment analysis results showed that F3H proteins have high homology. By NCBI-blasp amino acid sequence alignment, PeF3H showed 80 and 75% identity with F3H in *Arabidopsis. thaliana* (NP_190692.1) and *Oryza sativa* (NP_001054157.1), respetively, indicating the conservation of F3H in plants.

The evolutionary relationship of PeF3H with F3H in other plants was analyzed using MEGA11 software, and an evolutionary tree was constructed using the neighbor-joining alignment method. Results revealed that F3H is relatively conservative in evolution in monocotyledonous and dicotyledonous plants. PeF3H is closely related to a putative ATP-dependent RNA helicase an3 in *Turnera subulata* (KAJ4844570.1), and a naringenin, 2-oxoglutarate 3-dioxygenase-like in *Populus alba* (XP_0034889683.1), with 88% identities (Fig. 3). The highly conservative of F3H indicate that F3H gene plays an important role in the growth of the plant.

Sequence analysis of the amino acids encoded by the PeF3H gene revealed that the protein consists of 386 amino acid residues with a relative molecular mass of 43.2kDa, and a molecular weight of 43.2kDa with predicted isoelectric point (PI) of 6.38. The hydrophilicity/hydrophobicity analysis indicated that the majority of predicted amino acids were neutral (Fig.4A). Protein phosphorylation and dephosphorylation are involved in almost all activities in the cell life cycle. The predicted phosphorylation sites showed that PeF3H contained 13 serine sites, 5 threonine sites, and 2 tyrosine sites (Fig. 4B). The phosphorylation site on PeF3H protein may be phosphorylated by kinase, and then participate in the signal transduction process of regulating the growth or stress resistance of passion fruit.

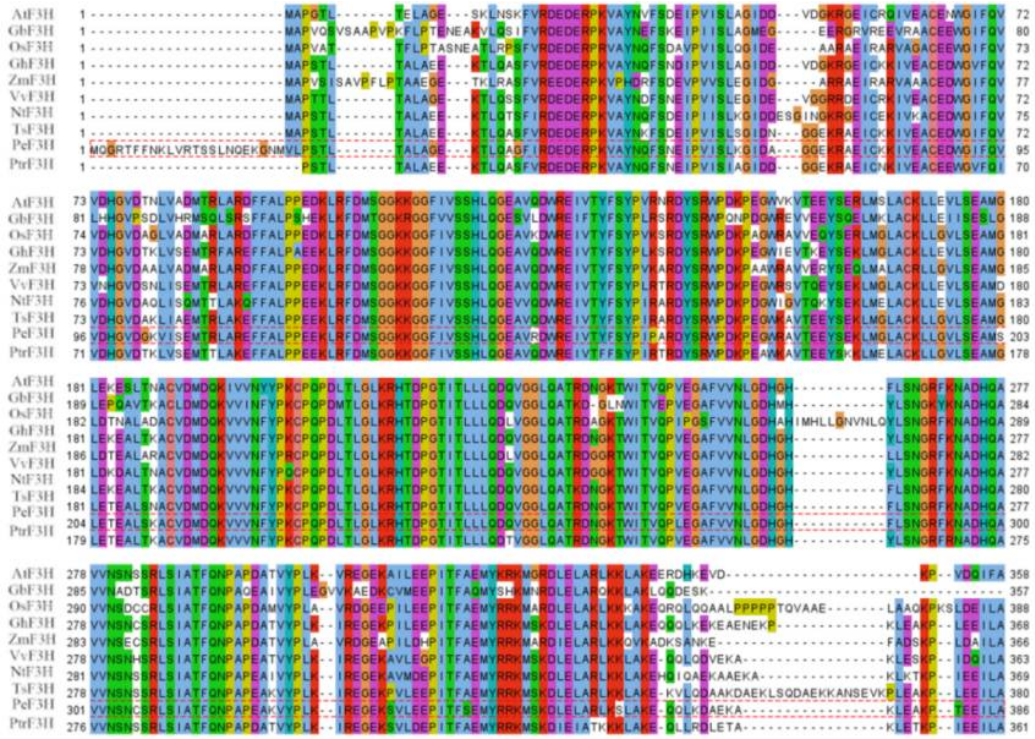

Fig. 2. The sequence difference alignment of PeF3H amino acid. AtF3H(NP190692.1), GbF3H(A AU93347.1), OsF3H(NP001054157.1). GhF3H(ABM64799.1). ZmF3H(NP001130275.1), VvF3H (NP001268 03 4.1), NtF3H(NP001312012.1), TsF3H(KAJ4844570.1), PeF3H(P_edulia040010337.g), PtrF3H(XP052308812. 1).

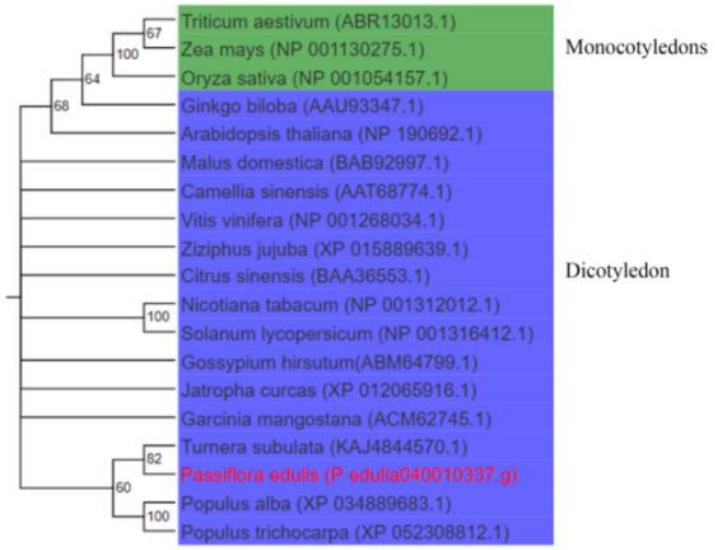

Fig. 3. Phylogenic analysis of F3H proteins in different plants.

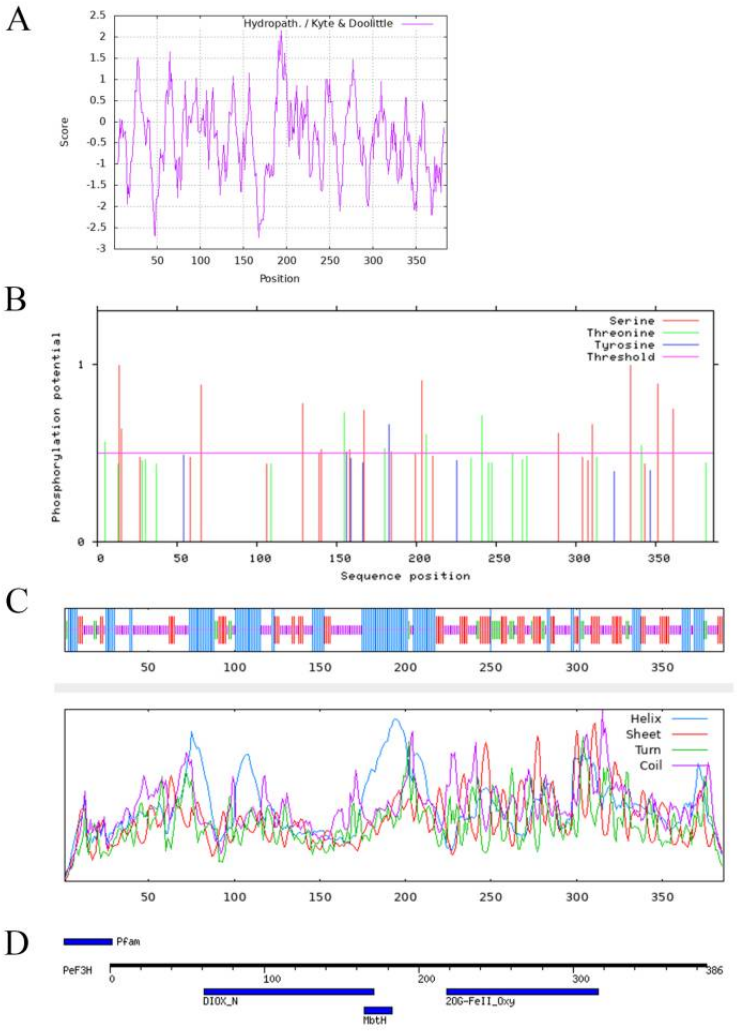

Fig. 4. Predicted physicochemical properties of PeF3H protein. (A) Hydrophilic and hydrophobic predication; (B) Phosphoricacid site predication; (C) Secondary structure prediction; (D) Motif prediction.

Moreover, the secondary structure of PeF3H analyzed results indicated that the alpha helix of the protein accounted for 29.79% (blue part), the extended strand and beta turn accounted for 21.76 % and 7.25% (red part), respectively, and the random coil accounted for 41.19% (orange part) (Fig. 4C). Finally, the Motif was predicted a DIOX_N binding region between sites 39-149 and 2OG-FeII_Oxy at sites 196-294, indicating the consistency of the PeF3H structure with that of F3H (Fig. 4D).

The subcellular localization of the PeF3H protein showed that a 69.6% chance localized in the mitochondria, 17.4% in the cytoplasm, and 13.0% in the nucleus, suggesting a potential mitochondrial localization for PeF3H. These bio-informatics analyses provide a foundation for further experimental studies on the function and structure of the PeF3H.

The three-dimensional (3D) structure of the PeF3H protein was predicted by SWISSMODEL and Alphafoold2. The predicted 3D structure of PeF3H is highly homology to a thebaine 6-O-demethylase (5o7y.1) determined by X-ray 2.0Å crystallography, with GMQE 0.63 and 30.27 identity by SWISSMODEL analysis. While, it was found that PeF3H had high homology to a Fe2OG dioxygenase domain-containing protein (A0A067LEF8) in *Jatropha curcas* by blastp, which have 88% identical amino acid sequences. The front and reverse views of the 3D structure model in carton and sphere shapes were shown in Fig.5A-D. In this 3D model, the beta turn in the center position of the protein formed a space, where flavanones added a -OH to form dihydroflavonols, which are precursors of various flavonoids and anthocyanin.

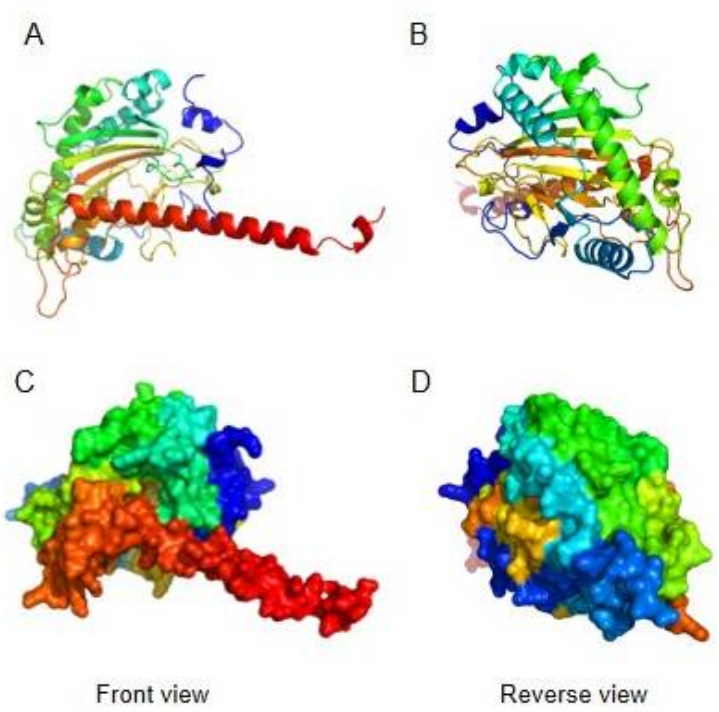

Fig. 5. Homology modeling of PeF3H (A0A067LEF8). (A) Front view, (B) Reverse view, (C) Front Spheres view, (D) Reverse Spheres view. Protein showed in rainbow color, blue at N terminal, red at C terminal.

To gain a deeper understanding of the regulatory relationships between PeF3H and other proteins, a protein-protein interaction (PPI) analysis was conducted based on the homology of the A0A067LEF8. Using the String software, the associative regulatory relationships between F3H and its interacting proteins were identified (Fig. 6). The PPI analysis revealed that F3H interacts with several other proteins, including two F3H, two DFR (dihydroflavonol 4-reductase), two P450 (cytochrome P450), one CHS (chalcone synthase), CHI (chalcone isomerase), F3’H (flavonoid 3’-hydroxylase), and F3’5’H (flavonoid 3’,5’-hydroxylase). These proteins are all associated with the flavonoid synthetic pathway and play important roles in the regulation of F3H's biological activity.

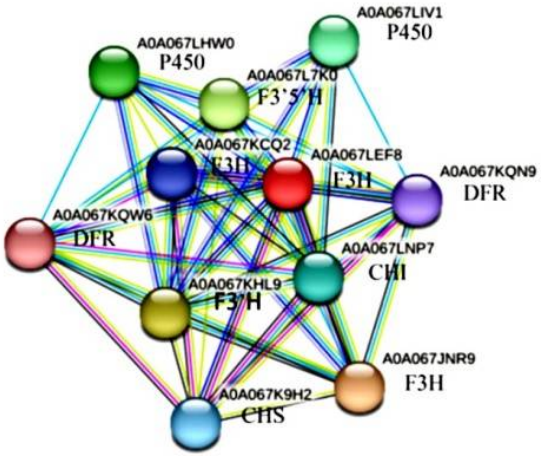

Fig. 6. Predicted PPI network of F3H.

The KEGG pathway analysis of PeF3H revealed its involvement in several important pathways, including ko00941 (Flavonoid biosynthesis), ko01100 (sedoheptulose-bisphosphatase), and ko01110 (phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase, PTEN). In the ko00941 Flavonoid biosynthesis pathway, F3H catalyzed naringenin, eridictyol, and dihydrotricetin to the corresponding compound with an addition of a hydroxyl group (-OH) at 3C. These results suggest that PeF3H plays a crucial role in the biosynthesis of flavonoids and is involved in several other important cellular processes, including sedoheptulose-bisphosphatase and PTEN-mediated signaling pathways (Fig. 7).

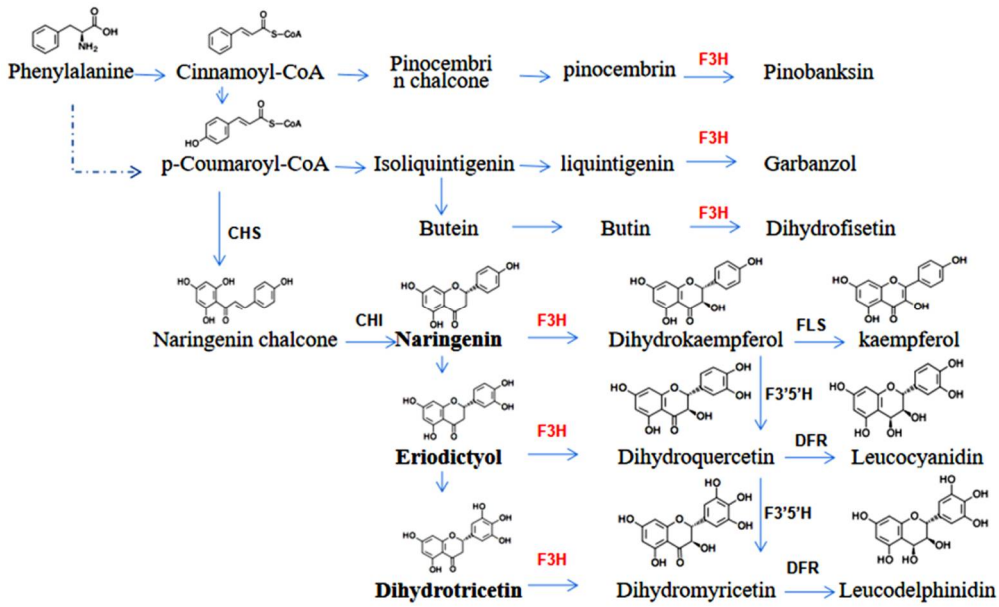

Fig. 7. KEGG pathway of F3H in flavonoid biosynthesis.

Results of quantitative real-time PCR (qRT-PCR) analysis demonstrated that the expression level of PeF3H was significantly higher in leaves, followed by stems and roots (Fig. 8). The differential expression of PeF3H in various tissues suggests that it is not a tissue-specific gene. The expression level of F3H is associated with the content of flavonoid and affect the function of growth and stress resistance of the plant.

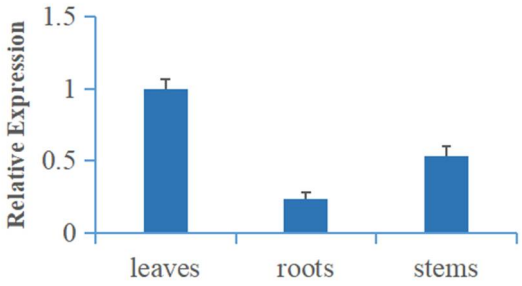

Fig. 8. Expression of *F3H* in different tissues part of passion fruit.

Polyphenolic secondary metabolites have gained recognition as natural products with unique physiological and pharmacological activities in the field of phytopolyphenolic chemistry and pharmacology (Song et al. 2000). The F3H gene is a key structural gene of the core flavonoid-anthocyanin pathway and has been receiving increasing attention (Yin *et al*. 2021). In the present study, it was possible to successfully clone the PeF3H gene using fresh leaf RNA from *P. edulis*. The PeF3H gene has a CDS sequence of 1161 bp, encoding 386 amino acids. Motif prediction analysis of the PeF3H protein showed that it contains a DIOX-N superfamily and a 2OG-FeII-Oxy oxygenase structural domain.

F3H is a key enzyme involved in the biosynthesis of flavonoids, a diverse group of plant secondary metabolites with plant growth, disease and stress resistance, quality, and color formation biological activities. F3H catalyzes the hydroxylation of flavanones to dihydroflavonols, which are important precursors of various flavonoids. F3H is a metalloenzyme that requires iron as a cofactor to catalyze the conversion of flavanone to dihydroflavonone. The 3D structure of F3H provides important insights into its catalytic mechanism and substrate specificity. The conserved amino acid sites His218, His276, Asp220, Arg286 and Ser288, in 2-ODD domain, can promote the activity of F3H (Liu *et al*. 2013). The crystal structure of F3H from *Petunia hybrida* shows that the protein forms a homodimer, with each monomer consisting of a conserved double-stranded beta-helix (DSBH) fold, which contains the active site residues and the 2OG-Fe (II) binding sites (Sun *et al*,, 2017). Docking with α-KG (2-ketoglutaric acid), Fe^{3+}, the binding sites of CtF3H1 was predicted, which can be referenced in the research of F3H enzyme engineering in plant (Sui *et al*. 2023).

The interaction of F3H with other proteins in the flavonoid biosynthesis pathway highlights the complex regulation of this important metabolic pathway. Several studies have identified proteins that interact with F3H, including transcription factors, enzymes, and regulatory proteins. For example, CHS, anthocyanidin synthase (ANS), F3H, flavonol synthase (FLS), and DFR have been reported to interact with F3H (Jan *et al*. 2021, Wang *et al*. 2021). In addition, the transcription factors MYB12 and MYB111 have been shown to interact with F3H to regulate flavonoid biosynthesis in *A. thaliana, Carya cathayensis*, and saffron (Stracke *et al*. 2007, Pathak *et al*. 2022, Wang *et al*. 2022). Furthermore, WRKY and NAC transcription factors have also been

shown to interact with F3H (Wang *et al*. 2017, Gao *et al*. 2021). By manipulating these transcription factors, it may be possible to increase or decrease flavonoid production in different plant species for various applications.

Accumulation of flavonoids has been shown to increase the tolerance of plants to biotic stress. Overexpression of F3H in *A. thaliana* led to increased levels of flavonoids. Overexpression of *CsF3H* provided tolerance to salt stress and fungus *Alternaria solani* infection to transgenic tobacco through an improved antioxidant system and enhanced pectin methyl esterification (Mahajan and Yadav 2014). Overexpression of F3H in rice showed increased accumulation of flavonoids, promoting plant growth and increasing tolerance to bacterial leaf blight (Jan *et al*. 2021). While the knockdown of F3H in citrus fruits resulted in decreased levels of flavonoids (Mou *et al*. 2021). By cloning F3H gene and over-expressing or knocking down the transgene in crops, one can obtain germplasm with different stress resistance or phenotype.

Understanding the molecular mechanisms involved in the biosynthesis and regulation of flavonoids may lead to the development of new strategies for enhancing the color, flavor, and nutritional value of passion fruit and other fruits and vegetables. In the present study, the sequence and protein structure of one F3H gene from *P. edulis* were analyzed bioinformatically, and the gene's expression in three tissues were also analyzed using qRT-PCR. Overexpression of the F3H gene in *P. edulis* may lead to increased flavonoid production, which may have potential commercial applications in the food and pharmaceutical industries.

Acknowledgments

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