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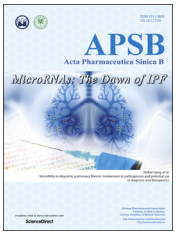

ORIGINAL ARTICLE

Cloning and expression of *SgCYP450-4* from *Siraitia grosvenorii*

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KEY WORDS

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CYP87A3;
Race clone;
Expression pattern

Abstract CYP450 plays an essential role in the development and growth of the fruits of *Siraitia grosvenorii*. However, little is known about the *SgCYP450-4* gene in *S. grosvenorii*. Here, based on transcriptome data, a full-length cDNA sequence of *SgCYP450-4* was cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid-amplification of cDNA ends (RACE) strategies. *SgCYP450-4* is 1677 bp in length (GenBank accession No. AEM42985.1) and contains a complete open reading frame (ORF) of 1422 bp. The deduced protein was composed of 473 amino acids, the molecular weight is 54.01 kDa, the theoretical isoelectric point (PI) is 8.8, and the protein was predicted to possess cytochrome P450 domains. *SgCYP450-4* gene was highly expressed in root, diploid fruit and fruit treated with hormone and pollination. At 10 days after treatment with pollination and hormones, the expression of Sg*CYP450-4* had the highest level and then decreased over time, which was consistent with the development of fruits of *S. Grosvenorii*. Hormonal treatment could significantly induce the expression of *SgCYP450-4*. These results provide a reference for regulation of fruit development and the use of parthenocarpy to generate seedless fruit, and provide a scientific basis for the production of growth regulator application agents.

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1. Introduction

Siraitia grosvenorii is an important traditional Chinese medicine and an endemic species of China. It has been reported to promote detoxification, prevent cough and sputum, sooth the stomach, clear the lungs and lubricate the intestine[1]. It is a unique medicinal plant only distributed in the mountainous areas in the northern of Guangxi in China[2]. Its active ingredient, mogrosides V, is a new sweetener with therapeutic benefits and has been drawing increasing attention. However, the production of this ingredient is limited by the difficulties in *S. grosvenorii* cultivation, including a requirement for heavy artificial pollination, scarcity of appropriate cultivatable land and the high purifucation cost due to the presence of seeds[3]. Parthenocarpy is an effective way to generate seedless fruits[4–7] and does not require artificial pollination. Seedless fruits, such as pear, eggplant, pimiento, cucumber, and tomato, have been obtained by treating plants with growth regulators[8].

Research has shown that auxin is an important factor for fruit development. Parthenocarpic fruits can be generated by regulating the key factors of auxin (acetic acid, IAA) biosynthesis and its signal transduction pathway. Researchers have obtained parthenocarpic fruits by the expression of the auxin biosynthesis enzyme indole acetic acid monooxygenase (iaaM)[9], CYP79B2 or CYP79B3[10], YUCCA[11] and inactivation of expression or mutation of auxin/indole-3-acetic 9 (IAA9) and auxin response factor 7 (ARF7, ARF8), and auxin signal transduction factors[12–14]. Decreased expression of *CYP87A3*, an early regulator gene in the auxin signaling pathway (Fig. 1), led to elevated auxin responsiveness of growth. A similar reduction of *CYP87A3* was found for a further mutant, hebiba, with elevated auxin responsiveness of growth and might be responsible for the decrease in growth[15]. The *CYP87A3* gene product might be directly involved in IAA de-activation/transport inhibition or alternatively in the biosynthesis of auxin antagonists. *CYP87A3* is only transiently increased (peak at 1 h) in response to auxin, possibly encoding a regulatory component involved in tissue adaptation to higher auxin concentration. A rapid return to the initial lower transcript level would restore the cellular competence for subsequent responses to changing auxin concentration[16]. These are different with several other early auxin response factors, including members of the SAUR[17–19], GH3[20] and AUX/IAA[21] families. Growing evidence has appeared concerning regulation of auxin action at the level of gene expression of *GH3* or *Aux/IAA*[22–24]. From the expression pattern of such genes, their role in signaling as well as their putative function has been inferred. But little research on *CYP87A3* has been reported. *SgCYP450-4* has the highest homology with *CYP87A3* and is still far from being understood in *S. grosvenorii*. Therefore, the full-length cDNA sequence of *SgCYP450-4* from *S. grosvenorii* was isolated and its expression pattern was investigated. The results may lay the foundation for further exploration of the biological means by which it is regulated, and reveal the underlying mechanism of the auxin signal transduction pathway in *S. grosvenorii.*

2. Materials and methods

2.1. Plant materials

S. grosvenorii cultivar 'Yongqing No. 1' (diploid fruits with seeds) and the 'Yaoyuanwuzi No. 1' (triploid fruits with seedless) were planted in the experimental farm of Guangxi Medicinal Botanical Garden, Nanning, China. All the plants and their fruits used in this study were breed and identified by Dr. Xiaojun Ma. There were five groups including A group [the different development period of seedless fruits: on the flowering day and 3 h, 1, 3, 5, 10 and 15 days after treated with growth regulators (the patent of our laboratory[25])], B group (10-day-old seedless fruits with different treatment: pollination, growth regulators and the pollination combined with growth regulators), C group (different development period of diploid fruits with seeds: 10, 20, 30, 40, 50, 60 and 70 days), D group (different tissues of the diploid plant: root, stem, leaf, flower, peel and pulp), and E group (40-day-old of diploid, triploid and tetraploid fruits). All the samples were collected and stored in liquid nitrogen for tissue expression analysis.

2.2. RNA isolation and synthesis of cDNA

Total RNA was extracted using the Trizol (Invitrogen, USA) method of Tang et al.[26]. Quality and quantity of each total RNA sample were assessed in agarose gels (1%, *w/v*) and spectrophotometrically at 260 and 280 nm (Bio-Rad, NanoDrop 2000), respectively. cDNA was synthesized with a reverse transcription kit (TaKaRa PrimeScript™, China) according to the manufacturer's instructions. The cDNA were stored at −20 °C for RT-qPCR analysis and gene analysis.

2.3. Cloning of SgCYP450-4 by RACE method

The primers used in this study are shown in Table 1. The first-strand cDNA was used as the template for *SgCYP450-4* core fragment amplification based on Unigene 454 data[26]. The PCR products were generated with a RACE kit (Clontech, USA) following the manufacturer's instructions and then subjected to electrophoresis on a 1% agarose gel for detection and purification. The selected amplified fragments were cloned into the pGM-Tvector (Tiangen). Recombinant plasmids were transformed into *Escherichia coli*, selected by blue/white screening, and verified by PCR. Nucleotide sequencing was performed by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., China.

2.4. PCR of ORF of sgCYP450-4

The primers are shown in Table 1. The PCR reaction contained 2 μL of cDNA, 2 μL of gene-specific primers, 25 μL of 2 × Trans Taq TM HiFi PCR Super Mix, 19 μL of double-distillated H_2O (dd H_2O), in a final volume of 50 μL. PCR was carried out as

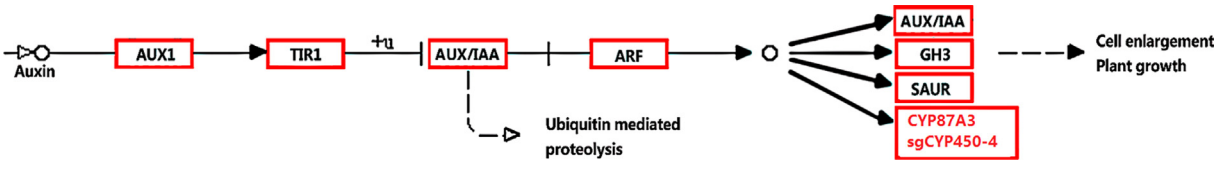

Figure 1 The signal transduction pathway of auxin.

Table 1 Primers for gene cloning and real-time PCR detection.

Gene name	Primer
5′ RACE *CYP450-4*	GTTTGGTCCATCGGTATTTGGTACAGAG
3′ RACE *CYP450-4*	CTCTGTACCAAATACCGATGGACCAAAC
5′ ORF	ATGTGGACTGTCGTGCTCGG
3′ ORF	TCATTCCTTGGGTGTGAACTTC
18s RNA	CTTCGGGATCGGAGTAATGA GCGGAGTCCTAGAAGCAACA
sgCYP450-4	TTTGTACTGCTGTCTTTGCTTCA GTTTGGAAGAGCATGGTTTTATT

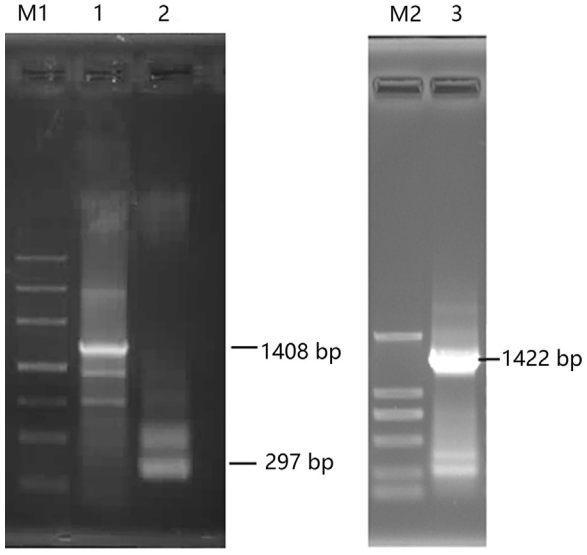

Figure 2 Amplification and detection of *SgP450-4* 5′-RACE, 3′-RACE and ORF. (M1) D4500 DNA marker; (1) 5′-RACE product; (2) 3′-RACE product; (M2) D2000 DNA marker; (3) open reading frame product.

follows: 94 °C for 5 min, 94 °C for 40 s, 50 °C for 40 s, 72 °C for 1 min, 35 cycles.

2.5. *Isolation and bioinformatics analysis of SgCYP450-4*

The physical and chemical properties of the SgCYP450-4 protein were predicted by the ExPASy Proteomicsm Server (http://www.Expasy.ch/tools/protparam.html), and the SWISS-MODEL[27–30] (http://swissmodel.Expasy.org/) predicted the protein secondary structure and analyzed the molecular modeling of SgCYP450-4. SignalP 4.0 Server (http://www.Cbs.Dtu.dk/services/SignalP/) and TargetP 1.1 Server (http://www.Cbs.Dtu.dk/services/TargetP/) were used to predict the signal peptide. SubLoc. v1.0[31] (http://www.bioinfo.tsinghua.edu.cn/SubLoc/) and TMHMM Server v.2.0 (http://www.Cbs.Dtu.dk/services/TMHMM/) were used to predict the protein subcellular localization and transmembrane region. Conserved domains were analyzed by Blastp and ExPASy PROSITE (http://www.Expasy.ch/prosite/). Finally, phylogenetic analysis was constructed for 13 proteins of different species using MEGA version 5.0 by the neighbor-joining method with 1000 bootstrap replicates to determine the relationship between CYP450-4 and other proteins.

2.6. *RT-qPCR of SgCYP450-4*

RNA was extracted from tissues of all samples and each sample was used for RT-qPCR. RT-qPCR was performed using an ABI 7500 fluorescence quantitative PCR instrument. Each reaction contained cDNA (1 μL), gene-specific primers (1 μmol/L), Rox 0.5 μL, ddH_2O 10 μL and 2 × SYBR Green Master Mix (12.5 μL) (SYBR® Premix Ex TaqTMII (Tli RNaseH Plus), TaKaRa PrimeScript™, China) in a final volume of 25 μL. The *18s RNA* gene was used as a reference gene[32,33]. The primer sequences are shown in Table 1. RT-qPCR analysis was carried out as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 95 °C for 5 s and 60 °C for 34 s. All experiments were done in triplicate for each sample and relative gene expression levels were calculated using the relative standard curve method. Each sample has three biological replicates and three techniques replicates with a negative control.

3. Results

3.1. *Molecular cloning of full-length cDNAs and characterization of SgCYP450-4*

Based on the sequences of unigenes from *S. grosvenorii* transcriptome data[26], a full-length cDNA clone was obtained using 5′-/3′-RACE extension methods. Two specific primers, 5′ RACE *CYP450-4* for 5′-RACE, and 3′ RACE *CYP450-4* for 3′-RACE were designed (Table 1) to yield a 1408 bp 5′-cDNA sequence and a 297 bp 3′-cDNA sequence (Fig. 2). Sequence analysis confirmed that the clone is an *SgCYP450-4* gene. The full-length cDNA is 1677 bp. Its ORF is 1422 bp (Fig. 3). The cloned cDNA has been submitted to GenBank under the accession number AEM42985.1.

3.2. *Bioinformatics and analysis of SgCYP450-4*

3.2.1. *Physical and chemical property analysis*

The SgCYP450-4 protein atomic formula is $C_{2479}H_{3866}N_{642}O_{677}S_{16}$, encoding a deduced protein of 473 amino acids with a predicted molecular weight of 54.01 kDa and an theoretical isoelectric point (PI) of 8.81. The total number of negatively charged residues (Asp+Glu) are 55, and there are 61 positively charged residues (Arg+Lys). The instability index is computed to be 35.37, which classifies this protein as stable. The aliphatic index is 94.61 and the grand average value of hydropathicity (GRAVY) is –0.119.

3.2.2. *Secondary structure prediction and three-dimensional model*

The secondary structure prediction shows that SgCYP450-4 contains 47.78% α-helix, 40.09% loop and 6.13% strand. A three-dimensional structural model was also constructed by SWISS-MODEL, with amino acids 31–472 used to establish the model with the 5e0e.1.A protein as the template, and described as the cytochrome P450 family 2 subfamily B. The sequence homology was 21.35% (Fig. 4).

```
  67 atgtggactgtcgtgctcggtttggcgacgctgtttgtcgcctactacatccattggattaacaaatggagagattccaagttcaacgga
     M  W  T  V  V  L  G  L  A  T  L  F  V  A  Y  Y  I  H  W  I  N  K  W  R  D  S  K  F  N  G
 157 gttctgccgccgggcaccatgggtttgccgctcatcggagagacgattcaactgagtcgactcagtgactccctcgacgttcacccttttc
     V  L  P  P  G  T  M  G  L  P  L  I  G  E  T  I  Q  L  S  R  L  S  D  S  L  D  V  H  P  F
 247 atccagaaaaaagttgaaagatacgggccgatcttcaaaacatgtctggccggaaggccggtggtggtgtcggcggacgcagagttcaac
     I  Q  K  K  V  E  R  Y  G  P  I  F  K  T  C  L  A  G  R  P  V  V  V  S  A  D  A  E  F  N
 337 aactacataatgctgcaggaaggaagagcagtggaaatgtggtatttggatacgctctccaaatttttcggcctcgacaccgagtggctc
     N  Y  I  M  L  Q  E  G  R  A  V  E  M  W  Y  L  D  T  L  S  K  F  F  G  L  D  T  E  W  L
 427 aaagctctgggcctcatccacaagtacatcagaagcattactctcaatcacttcggcgccgaggccctgcgggagagatttcttcctttt
     K  A  L  G  L  I  H  K  Y  I  R  S  I  T  L  N  H  F  G  A  E  A  L  R  E  R  F  L  P  F
 517 attgaagcatcctccatggaagcccttcactcctggtctactcaacctagcgtcgaagtcaaaaatgcctccgctctcatggtttttagg
     I  E  A  S  S  M  E  A  L  H  S  W  S  T  Q  P  S  V  E  V  K  N  A  S  A  L  M  V  F  R
 607 acctcggtgaataagatgttcggtgaggatgcgaagaagctatcggaaatatccctgggaagttcacgaagcttctaggaggatttctc
     T  S  V  N  K  M  F  G  E  D  A  K  K  L  S  G  N  I  P  G  K  F  T  K  L  L  G  G  F  L
 697 agtttaccactgaattttcccggcaccacctaccacaaatgcttgaaggatatgaaggaaatccagaagaagctaagagaggttgtagac
     S  L  P  L  N  F  P  G  T  T  Y  H  K  C  L  K  D  M  K  E  I  Q  K  K  L  R  E  V  V  D
 787 gatagattggctaatgtgggccctgatgtggaagatttcttggggcaagcccttaaagataaggaatcagagaagttcatttcagaggag
     D  R  L  A  N  V  G  P  D  V  E  D  F  L  G  Q  A  L  K  D  K  E  S  E  K  F  I  S  E  E
 877 ttcatcatccaactgttgttttctatcagttttgctagctttgagtccatctccaccactcttactttgattctcaagctccttgatgaa
     F  I  I  Q  L  L  F  S  I  S  F  A  S  F  E  S  I  S  T  T  L  T  L  I  L  K  L  L  D  E
 967 cacccagaagtagtgaaagagttggaagctgaacacgaggcgattcgaaaagctagagcagatccagatggaccaattacttgggaagaa
     H  P  E  V  V  K  E  L  E  A  E  H  E  A  I  R  K  A  R  A  D  P  D  G  P  I  T  W  E  E
1057 tacaaatccatgacttttacattacaagtcatcaatgaaacccctaaggttggggagtgtcacacctgccttgttgaggaaaacagttaaa
     Y  K  S  M  T  F  T  L  Q  V  I  N  E  T  L  R  L  G  S  V  T  P  A  L  L  R  K  T  V  K
1147 gatcttcaagtaaaaggatacataatcccggaaggatggacaataatgcttgtcaccgcttcacgtcacagagacccaaaagtctataag
     D  L  Q  V  K  G  Y  I  I  P  E  G  W  T  I  M  L  V  T  A  S  R  H  R  D  P  K  V  Y  K
1237 gaccctcatatcttcaatccatggcgttggaaggacttggactcaattaccatccaaaagaacttcatgccttttgggggaggcttaagg
     D  P  H  I  F  N  P  W  R  W  K  D  L  D  S  I  T  I  Q  K  N  F  M  P  F  G  G  G  L  R
1327 cattgtgctggtgctgagtactctaaagtctacttgtgcaccttcttgcacatcctctgtaccaaataccgatggaccaaacttgggggga
     H  C  A  G  A  E  Y  S  K  V  Y  L  C  T  F  L  H  I  L  C  T  K  Y  R  W  T  K  L  G  G
1417 ggaaggattgcaagagctcatatattgagttttgaagatgggttacatgtgaagttcacacccaaggaatga 1488
     G  R  I  A  R  A  H  I  L  S  F  E  D  G  L  H  V  K  F  T  P  K  E  *
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Figure 3 Open reading frame (ORF) and deduced amino acid sequence of *SgCYP450-4*. The 67–1488 bp ORF contained 1422 bp; the deduced protein was composed of 473 amino acids.

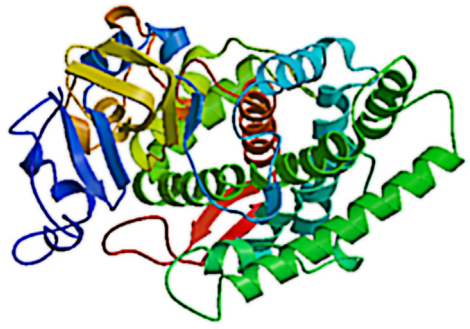

Figure 4 Three-dimensional structure of SgCYP450-4.

3.2.3. Signal peptide, subcellular localization and transmembrane region prediction

The Signal P 4.0 Server predicted nuclear localization signals indicating that the SgCYP450-4 protein has no signal peptide, but the protein contains a highly conserved leading peptide by TargetP v.1.1 Server, predicting that it localized in the chloroplast, as well as a mitochondrial targeting peptide (mTP, 0.049), a secretory pathway signal peptide (SP, 0.928) and other peptide (0.133), the predict credibility of which was 2. To predict the subcellular localization of SgCYP450-4 protein by Subloc v. 1.0, the results showed that SgCYP450-4 protein localized in the cytoplasm most likely; the reliability index (RI) is 3 and the expected accuracy is 84%. The results of TMHMM Server v. 2.0 to predict the transmembrane region of SgCYP450-4 showed that the protein has no transmembrane region, suggesting it is a secreted protein rather than a membrane-associated protein.

3.2.4. Domains prediction

Searching for the conserved domains in the protein using the NCBI Conserved Domain Database and InterProScan online tools showed that SgCYP450-4 contains the p450 (33–453) domain, CypX (127–472) domain, P450-cycloAA-1 (214–439) domain and the P450-rel-GT-act (340–399) domain, belonging to the cytochrome P450, cytochrome P450 (secondary metabolites biosynthesis, transport and catabolism, defense metabolism), and cytochrome P450 (cyclodipeptide synthase-associated, members of this subfamily are cytochrome P450 enzymes that occur next to tRNA-dependent cyclodipeptide synthases), respectively, and contains PLNO2774 (4–471, a member of the superfamilly cl2078) and PLNO2738 (300–424, carotene β-ring hydroxylase) multiple domains. While the prediction of InterProScan showed that there were 4 types in total 8 conserved domains (Fig. 5), including cytochrome P450 domain (IPR001128) which consists of SSF48264, PF00067 and G3DSA; cytochrome P450, E-class, group 1 (IPR002401) which consists of PR00463 (EP4501); cytochrome P450, conserved site (IPR017972) which consists of PS00086, and one type has no unintegrated signatures in IPR which consists of PTHR24286, PTHR24286 (SF74, SUB) and TMhelix (Fig. 5).

SgCYP450-4 protein contains the cytochrome P450 domain and cytochrome P450, E-class, group 1 domain. Cytochrome P450s are haem-thiolate proteins involved in the oxidative degradation of various compounds. They are particularly well known for their role in the degradation of environmental toxins and mutagens. While prokaryotic P450s are soluble proteins, most eukaryotic P450s are associated with microsomal membranes, and their general enzymatic function is to catalyze regiospecific and

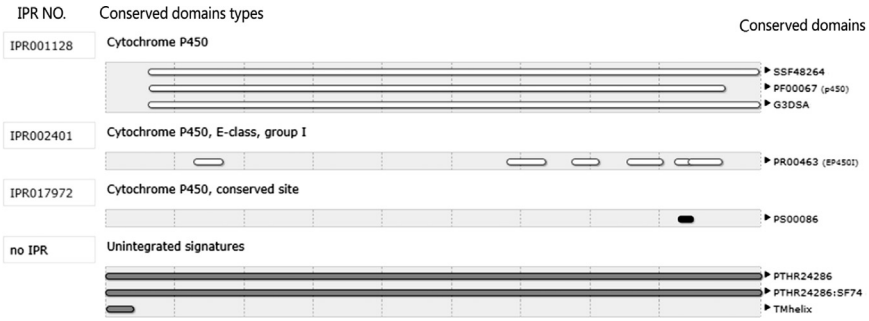

Figure 5 The conserved domains of SgCYP450-4 protein predicted by InterProScan. The conserved domains contained 4 types (cytochrome P450 domain; cytochrome P450, E-class, group 1; cytochrome P450, conserved site and unintegrated signatures) in total 8 conversed domains (SSF48264, PF00067, G3DSA, PR00463, PS00086, PTHR24286, PTHR24286 (SF74, SUB) and TMhelix).

stereospecific oxidation of non-activated hydrocarbons at physiological temperatures.

3.2.5. Amino acid sequence alignment and phylogenetic tree analysis

To determine the evolutionary relationship between SgCYPP450-4 from *Siraitia grosvenorii* and other species, a BLAST protein alignment and neighbor-joining tree were constructed for further identifying the relationships between the SgCYP450-4 and other related protein sequences from 12 plants already obtained. As shown in Figs. 6 and 7, SgCYP450-4 lined up with *Cucumis melo* (CmCYP83A1, XP_ 008437370. 1, identity was 83%) and *Cucumis sativus* CYP83A1 (XP 001292621. 1, 83%), followed by *Prunus mume* CYP83A1 (XP_008235946.1, 57%), which indicted that both proteins had similar structures and likely shared some gene functions.

3.3. The expression of SgCYP450-4 gene in different developmental periods

In the diploid fruits *SgCYP450-4* gene expression was at the highest level in the fruits of 10 days after pollination, and then had a sharp decrease after 20 and 30 days, respectively, and maintained a lower level of expression during days 40–70 (Fig. 8A). In the triploid fruits, *SgCYP450-4* gene expression was at the highest level in the fruits 10 days after pollination, but sharply decreased after 15 days (Fig. 8C).

3.4. Tissue-specific expression of SgCYP450-4 gene

To initially elucidate the function of the *SgCYP450-4* gene, we analyzed the expression pattern of the *SgCYP450-4* in root, stem, leaf, flower, peel and fruit of 40-day-old plants using the RT-qPCR technique. The results showed that *SgCYP450-4* was constitutively expressed in all tested tissues, but at very different levels. The transcription of *SgCYP450-4* gene was the highest in root, moderate in stem and the weakest in peel (Fig. 8B). The highest level of transcript was in root and was more than 10 times higher than in leaf.

3.5. The expression of SgCYP450-4 gene after different treatments

To examine the response of *SgCYP450-4* to hormones in our study, the level of *SgCYP450-4* transcript was analyzed using the RT-qPCR method in the samples treated with pollination combined with hormones, hormones only, and pollination only. Results showed that *SgCYP450-4* was positively and significantly induced by hormonal treatment. Generally, the expression level in fruit treated with pollination combined with hormones was 6 fold higher than that in the other two treatments, followed by the fruit treated with hormones only, and the lowest in the fruit treated with pollination only (Fig. 8D).

3.6. The expression of SgCYP450-4 gene in different ploidy fruits

The *SgCYP450-4* gene expression was the highest in diploid fruit, followed by triploid fruit, and the lowest expression in tetraploid fruit. The highest transcript of *SgCYP450-4* measured in diploid fruit was more than 3 times higher than in triploid fruit (Fig. 8E), indicating that the gene may be involved in the fruit development of diploid of *S. grosvenorii*, but the specific molecular mechanism needs further study.

4. Discussion

In plants, the *CYP87A3* gene has been cloned and characterized from rice[16], but not from *S. grosvenorii*. The CYP87A3 protein is most likely a regulator for the auxin responsiveness of growth. The transient regulation of this gene is accompanied by a change of endogenous auxin[15], which might be responsible for the growth. The *CYP87A3* gene product might be directly involved in IAA deactivation/transport inhibition or, alternatively, in the biosynthesis of auxin antagonists. Taking into account that CYPs are prominent enzymes in flavonoid biosynthesis, differential CYP87A3 expression might contribute to local distribution of specific flavonoids. Because flavonoids seemed to participate in regulation of auxin transport/oxidation, *CYP87A3* could be involved in the control of local auxin levels[34,35]. Therefore, to express recombinant functional CYP87A3 to test its effect on IAA oxidation or to screen for its potential substrate can help us to uncover the further function of CYP87A3. Here, we report the *sgCYP450-4* cDNA cloning and expression. SgCYP450-4 has the highest sequence similarity with the auxin early response factor CYP87A3-1 (83%) and may play an important role in auxin signal transduction. The deduced amino acid sequence of SgCYP450-4 showed extensive similarity to its counterparts in other species. In this study, based on the unigene sequence of *CYP450-4*, we designed specific primers and cloned the full-length cDNA sequence from *Siraitia grosvenorii*, named

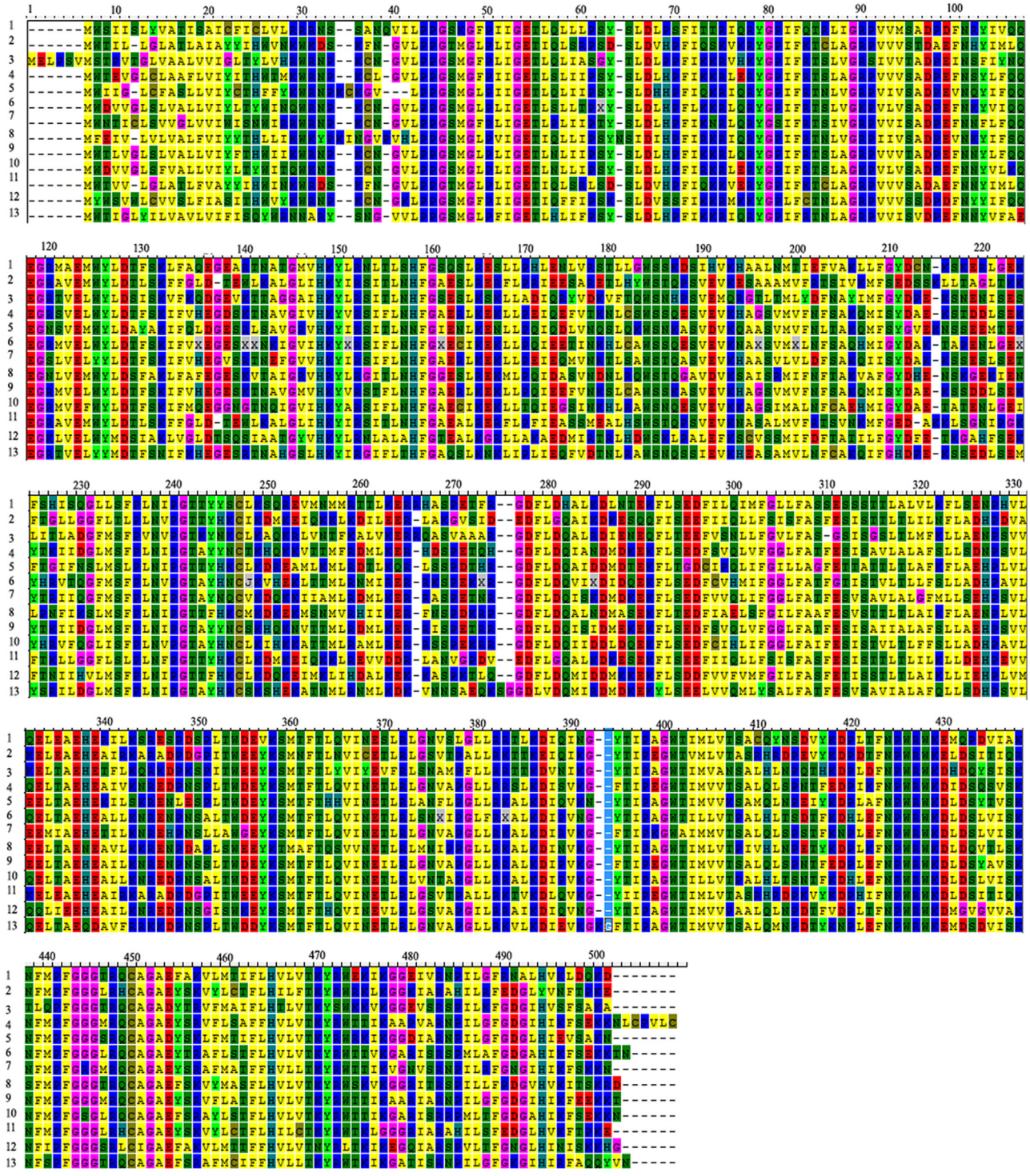

Figure 6 Homology comparison of amino acid sequence of *S. grosvenorii CYP450-4* with other plants. (1) *Beta vulqaris subsp. vulqaris CYP87A3* (XP_ 010690665.1); (2) *Cucumis melo CYP87A3* (XP_ 008437370.1) ; (3) *Cucumis sativus CYP87A3* (XP_ 001292621.1) ; (4) *Fragaria vesca subsp. vescal CYP87A3* (XP_ 004290050. 1) ; (5) *Jatropha curcas CYP87A3* (XP_ 012065519.1) ; (6) *Malus donestica CYP87A3* (XP_ 008364406.1) ; (7) *Morus notabills CYP87A3* (XP_ 010111721.1) ; (8) *Populus euphratica CYP87A3* (XP_ 011025040.1); (9) *Prunus mune CYP87A3* (XP_ 008235946.1) ; (10) *Pyrus X bretschneideri CYP87A3* (XP_ 009344155.1) ; (11) *S. grosvenorii CYP450-4* (AEM42985.1) ; (12) *Vitis vinifera CYP87A3* (XP_ 002272031.1) ; (13) *Ziziphus jujuba CYP87A3* (XP_ 015893049.1).

SgCYP450-4. The deduced SgCYP450-4 protein was observed to contain CYP450 domains, as does the CYP87A3 protein family. Multiple alignment analysis showed that SgCYP450-4 had more than 83% sequence identity with the CYP87A3 proteins of several other species, which suggests that SgCYP450-4 proteins are highly conserved, confirming the high degree of CYP87A3 conservation during evolution, which reflects the selective pressure imposed by the essential functions of CYP87A3 in plants.

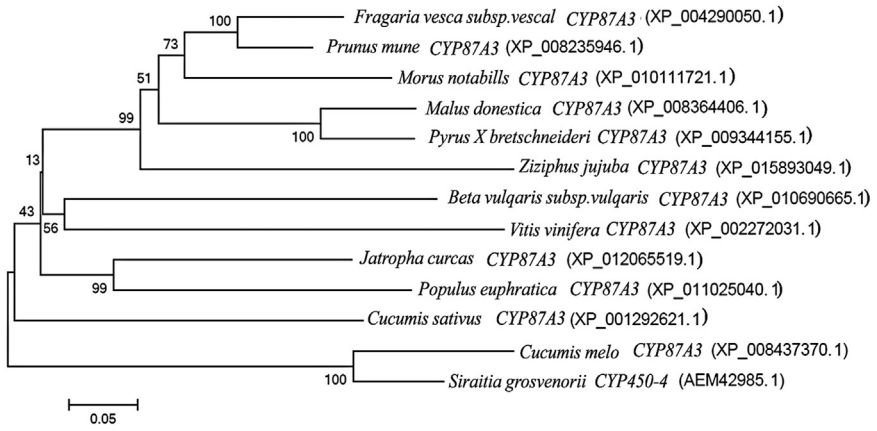

Figure 7 Phylogenetic analysis of SgCYP450-4 and other CYP87A3. *S. Grosvenorii* CYP450-4 lined up with *Cucumis melo* CYP87A3.

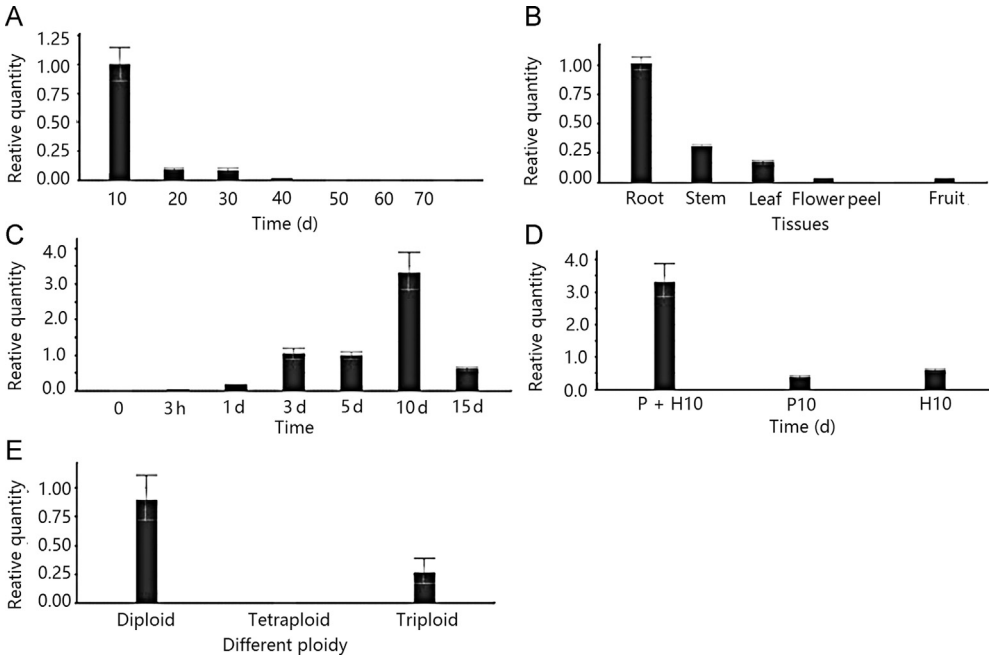

Figure 8 Expression patterns of *SgCYP450-4* in different samples of *S. grosvenorii*. (A) The expression of *SgCYP450-4* gene during different developmental periods in diploid fruit. (B) Tissue-specific expression of *SgCYP450-4* gene. (C) Expression of *SgCYP450-4* gene in different developmental periods in triploid fruit. (D) Expression of the *SgCYP450-4* gene with different treatments (P: pollination; H: hormones; P+H: pollination combined with hormones). (E) Expression of *SgCYP450-4* gene in different ploidy fruits.

Auxin has been considered to be the most important endogenous hormone in fruit growth, and especially in parthenocarpy fruit, auxin concentration is very high in many plant parthenocarpic ovaries. Auxin can promote cell division and elongation, and induce parthenocarpic fruit development. Gao et al.[36] generated a seedless tomato by treating with naphthylacetic acid (NAA). Auxin is closely associated with fruit setting and expansion. A certain concentration of auxin promotes ovary elongation to form the parthenocarpic fruit. IAA content increased after fertilization which resulted in the expansion of the ovary and surrounding tissue to accelerate the development of the fruit. With the addition of IAA to the ovary without fertilization, we also can obtain the parthenocarpic fruits of some plants, such as the pepper, watermelon, tomato, eggplant, holly, zucchini and fig, all obtained by spraying IAA on the stigmas before pollination[37,38]. The hormone level of fruit is low before pollination but significantly increased after pollination or other stimulation. Synthesis of IAA increases active gibberellins (GA) to promote the development of seeds and fruits. Even treating with the *N*-1-naphthylphthalamic acid (NPA, an auxin transport inhibitor) on the ovary without pollination can increase IAA content and result in parthenocarpy with higher GA content and GA biosynthesis, including ent-kuarenoic acid oxidase (*KAO*) and *GA20ox*[39]. The growth and development of fruits were affected by regulating the key factors in auxin biosynthesis and signal transduction. For example, the silencing or mutation of *IAA9* and *ARF7*, *ARF8* of auxin signal transduction factors could result in parthenocarpy fruits by affecting auxin signal transduction.

The S*gCYP450-4* gene had the highest expression level in 10-day-old seedless fruits and then maintained the low level with slow growth, which was consistent with the research of Mo et al.[40], that fruits grew slower after 10 days and resulted in the smaller fruits at maturity. While the *SgCYP450-4* gene expression level was low after 40 days in the fruits with seeds, suggesting that there was little change of fruit size after 40 days, and the same with the results of Jiang et al.[41] and Huang et al.[42] that the horizontal and vertical diameter of the *S. grosvenorii* fruit stopped growing after 40 days. *SgCYP450-4* had the highest expression level in diploid fruit, but low in triploid and tetraploid fruits. This may be the reason why triploid and tetraploid fruits are smaller than diploid fruit. As with the research of Mo et al.[40], the reason the triploid seedless fruits of *S. grosvenorii* were smaller was embryo abortion due to the poor fertilization, resulting in the low content of IAA and a serious lack of IAA in the ovary, preventing normal division and enlargement. Treatment with hormones combined with pollination increased the expression of *SgCYP450-4 gene,* indicating that spraying a certain concentration of growth regulator after 10 days may increase the fruit size.

CYP450s have a wide range of biological activities in plants[43,44], including the key synthase of IAA (CYP79B2, CYP79B3 and CYP83B1)[13] and auxin signal transduction response factor (CYP87A3), GA key synthase (ent-kaurene oxidase KO and ent-kaurenoic acid oxidase KAO were the members of CYP450)[45,46], the key synthase of mogrosides, such as oat root saponin (CYP51H10)[47], licorice triterpenoid saponin (CYP88D6, CYP93E3 and CYP72A154)[48,49] and *Medicago truncatula* saponin (CYP716A12)[50]. In the mogrosides synthesis pathway, CYP450 plays an important role in converting the *Cucurbita* dienol into mogrol, which is the precursor substance of mogrosides of *S. grosvenorii*, but which member of CYP450 family plays the key role remains to be determined. There were many transcripts of *CYP450* in the transcriptome of *S. grosvenorii*, but so far little biological function was determined. More researches on the *CYP450s* need to be done.

5. Conclusions

We cloned a *CYP450* gene from *S. grosvenorii* designated *SgCYP450-4*. According to the experimental results, the full-length ORF of *SgCYP450-4* is 1422 bp, encoding 473 amino acids with a predicted molecular weight of 50.01 kDa and an PI of 8.8. The *SgCYP450-4* gene is mainly expressed in root and stem, and the lowest in peel. The expression of *SgCYP450-4* could be significantly induced by hormone treatment. This work may lay a theoretical and experimental foundation for future research on gene functions, and the transgenic *S. grosvenorii* with varied *SgCYP450-4* expression will give deeper insight into the role of *SgCYP450-4* in *S. grosvenorii.*

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