Cloning of *MYB10, PAL* and *UFGT* genes from ‘Cuihongli’ and ‘Qiangcuili’

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Abstract. *PAL*, *UFGT* as structural genes and *MYB10* as a regulatory gene play an important role in the accumulation of anthocyanins in plants. In this experiment, 'Cuihongli' and 'Qiangcuili' were used as materials to clone *PAL*, *UFGT* and *MYB10* related to anthocyanin synthesis by homologous sequence cloning. The results showed that the full length of *PAL* was 2160 bp, encoding 719 amino acids; the full length of *UFGT* was 1428 bp, encoding 475 amino acids, and the differences of PAL and UFGT between the two cultivars were one amino acid and three amino acids, respectively. The sequence length of 'Cuihongli' *MYB10* gene is 732 bp, which encodes 243 amino acids and belongs to the superfamily of SANT family. Homology analysis and phylogenetic tree analysis showed that the proteins encoded by *PAL*, *UFGT* and *MYB10* genes were short and closely related to *Prunus persica*, *Prunus avium*, *Prunus armeniaca* and *Prunus cerasifera* in Rosaceae.

1. Introduction

The genes controlling the synthesis of anthocyanin are divided into structure genes and regulatory genes, the structural genes directly encode the enzyme which synthesize anthocyanin, and the expression intensity of these genes is related by the transcription factors[1]. Richard *et al*. found that *MdMYB10* isolated from apple had a positive regulatory effect on the formation of fruit color[2]. With the further study of the authors, it was also found that the repetition of a promoter fragment led to the automatic adjustment of *MYB10* transcription factors in red pulp apple, resulting in the accumulation of anthocyanins[3]. *PAL* is an enzyme that links biological primary metabolism with phenylalanine metabolism and catalyzes the first step of phenylalanine metabolism. It is the key enzyme and rate-limiting enzyme of phenylalanine metabolism[4]. Its catalytic reaction provides precursors for the synthesis of anthocyanins and other substances[5]. The activity of *PAL* enzyme in purple leaf increased int the light, which promoted the synthesis of anthocyanin[6]. *UFGT* mainly converts unstable anthocyanins into stable anthocyanins[7]. Glycosylation of anthocyanin molecules not only plays an important role in changing the color of plant flowers and maintaining the stability of molecular structure, but also is beneficial to the transport of anthocyanin molecules to vacuoles[7]. Kobayashi *et al*.[8] found that the expression of *UFGT* gene in grape berries played an important role in anthocyanin biosynthesis. It was found that there was a good positive correlation between *UFGT* expression and anthocyanin content in peach blossoms and leaves[7]. ‘Cuihongli’ is selected from the offsprings of Chinese plum(*Prunus salicina* Lindl), found in Sichuan. When the fruit ripens, the peel is purplish red and the pulp is yellow-green[9]. ‘Qiangcuili’ is one of the best plum varieties in Maoxian, Sichuan Province. From mid-July to late August, the fruit is ripe with green peel and yellow pulp. Researches shown that the red and purple-black color of the varieties is mainly determined by the content of anthocyanin[10, 11]. At present, the research on the ‘Cuihongli’ and ‘Qiangcuili’ in China has concentrated on its high-quality and high-yield cultivation technology[12], but the research on the molecular mechanism of fruit coloring is less. To explore the molecular mechanism of coloring difference between the two plum varieties, the regulatory gene *MYB10* and structural gene *PAL,UFGT* related to anthocyanin synthesis were cloned and analyzed in this experiment.

2. Materials and Methods

2.1. Materials

5-year-old 'Cuihongli' (purple-red peel and yellow pulp) and 'Qiangcuili' (yellow-green peel and pulp) were planted in Yanmen Township, Wenchuan County, Aba Tibetan Qiang Autonomous Prefecture, Sichuan Province(latitude 31 °28'34'N, longitude 103 °37'18'E, altitude 1460 m). The row spacing of the plant is 3 m × 3

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m, the cultivation and management are basically the same. 'Cuihongli' was sampled on June 2nd(Young fruit period), June 22nd(the initial stage of expansion), July 12nd(the rapid expansion period), August 1st(the initial stage of coloring), August 21st(the half-red stage), August 31st(the full-red stage) and September 10th(the maturation period), respectively. 'Qiangcuili' was sampled on June 2st(Young fruit period), June 22nd(the initial stage of expansion), July 12nd(the rapid expansion period), August 1st(initial stage of maturation) and August 21st(the maturation period), respectively. The experiment was carried out by randomized blocks design, fruits at the same height in the east, south, west and north of the tree was selected and brought back to the laboratory in the ice box.The peel was separated from the pulp. After the liquid nitrogen treatment, it was stored in the cryogenic refrigerator at -80 ℃ for total RNA extraction and cloning.

2.2. Methods

The extraction of total RNA refers to the CTAB method of Xu Qiuhong[13] and slightly modified. The synthesis of the first strand cDNA is carried out with reference to the PrimeScript ™ RT-agent Kit with gDNA Eraser (Perfect Real Time) kit of TaKaRa. cDNA from the same variety were mixed as the template for cloning. The sequence information of the *PAL*, *UFGT* and *MYB10* genes of related species of ‘Cuihongli’ and ‘Qiangcuili’ was searched on NCBI, and the primers were designed by Primer premier 5.0 software. The primer is synthesized by Bioengineering (Shanghai) Co., Ltd., and the sequence is shown in Table 1. The phylogenetic trees of proteins were constructed by MEGA5.10 software.

Table 1. Primer sequences for clone

Primer	Nucleotide of sequences (5′-3′)
kMYB10F	ATGGAGGGWTATAACTTGGGTGTGAGAAAAGGAGC
kMYB10R	CTATTCTTCWTTTGAATGATTCCAAAGGTCCACGC
PsPALF	ATGGAAATCGGCAATAAGG
PsPALR	GCSATAACCAGCACTCTCTA
PsUFGTF	TATATGGCACCRCAACCGAT
PsUFGTR	AAGTACAGCTCGGTTATTCT

3. Results and Discussion

3.1. Acquisition of MYB10 of ‘Cuihongli’

The total RNA reverse transcriptional cDNA of 'Cuihongli' and 'Qiangcuili' was used as template for PCR amplification. The amplified product was detected by 1% agarose gel electrophoresis. Only 'Cuihongli' obtained a target band. The fragment was purified and the gene sequence with length of 732 bp was obtained after sequencing. The biological information was analyzed by Protparam software. The full length of the protein encoded by 'Cuihongli' MYB10 gene was 3942 atoms, and the molecular weight was 28.40192 kD. The conserved domain of 'Cuihongli' MYB10 protein was analyzed by NCBI Conserved Domains database online analysis software, which belonged to SANT superfamily and contained several active sites.

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          10        20        30        40        50        60        70        80        90
1         ATGGAGGGTTATAACTTGGGTGTGAGAAAAGGAGCTTGGACTAGAGAGGAAGATGATCTTCTGAGGCAGTGCATTGAGAAACAAGGAGAA
1          M  E  G  Y  N  L  G  V  R  K  G  A  W  T  R  E  E  D  D  L  L  R  Q  C  I  E  K  Q  G  E

         100       110       120       130       140       150       160       170       180
91        GGAAAGTGGCACCAAGTTCCTTACAAAGCAGGATTAAGCAGATGCAGGAAGAGCTGTAGACTAAGGTGGTTGAACTATTTGAAGCCAAAT
31         G  K  W  H  Q  V  P  Y  K  A  G  L  S  R  C  R  K  S  C  R  L  R  W  L  N  Y  L  K  P  N

         190       200       210       220       230       240       250       260       270
181       ATCAAGAGAGGAGACTTTATGGAAGATGAAGTAGATCTAATAATTAGGCTTCACAAGCTTTTAGGAAACAGGTGGTCATTGATTGCTCGA
61         I  K  R  G  D  F  M  E  D  E  V  D  L  I  I  R  L  H  K  L  L  G  N  R  W  S  L  I  A  R

         280       290       300       310       320       330       340       350       360
271       AGACTTCCGGGAAGGACTGCCAATGATGTGAAAAATTACTGGAACACCCGATTGCGGACGGATTATTGCATGAAAAAGATGAAAGACAAA
91         R  L  P  G  R  T  A  N  D  V  K  N  Y  W  N  T  R  L  R  T  D  Y  C  M  K  K  M  K  D  K

         370       380       390       400       410       420       430       440       450
361       TCCCAAGAAACAATAAAGACCATAATAAGGCCACAACCAAGAAGATTCACCAAAAGTTCAAATTGTTTGAGTTTTAAAGAACCAATTTTG
121        S  Q  E  T  I  K  T  I  I  R  P  Q  P  R  R  F  T  K  S  S  N  C  L  S  F  K  E  P  I  L

         460       470       480       490       500       510       520       530       540
451       GACCATACTCAACTAGAAGAGAATTTTAGTACGACATCACAAACATCAACATCAACAAGGATTGGAAGTGATTGGTGGGAGACCTTTTTA
151        D  H  T  Q  L  E  E  N  F  S  T  T  S  Q  T  S  T  S  T  R  I  G  S  D  W  W  E  T  F  L

         550       560       570       580       590       600       610       620       630
541       GATGACAAGGATGCTACTGAAACAGCTACAGGTTCTGGTCTTGGGTTAGATGAAGAACTGCTCGCAAGTTTTTGGGTTGATGATGATATG
181        D  D  K  D  A  T  E  T  A  T  G  S  G  L  G  L  D  E  E  L  L  A  S  F  W  V  D  D  D  M

         640       650       660       670       680       690       700       710       720
631       CCACAATCGACAAGAACATGCGTCAATTTTTCTGAGGAAGGATTAAGTAGAGGTGATTTCTCTTTTAGCGTGGACCTTTGGAATCATTCA
211        P  Q  S  T  R  T  C  V  N  F  S  E  E  G  L  S  R  G  D  F  S  F  S  V  D  L  W  N  H  S

         730
721       AAAGAAGAATAGA
241        K  E  E  *
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Fig. 1 Sequence of the MYB10 Gene and the deduced amino acid sequence in 'Cuihongli'

3.2. Acquisition of PAL and UFGT of 'Cuihongli' and 'Qiangcuili'

The PAL sequence of the 'Cuihongli' and 'Qiangcuili' had a complete open reading frame of 2160 bp, encoding 719 amino acids. The two amino acid sequences were 99.9% the same, but the amino acids at position 102 were different (Val-Asp)(Fig. 2). The UFGT sequence of 'Cuihongli' and 'Qiangcuili' obtained a complete open reading frame of 1428 bp, encoding 475 amino acids. The consistency of the two amino acid sequences was 99.4%. There were differences between the amino acids at position 93, 329 and 458 (Asp-Glu, Asp-Glu, Ser-Pro)(fig. 3).

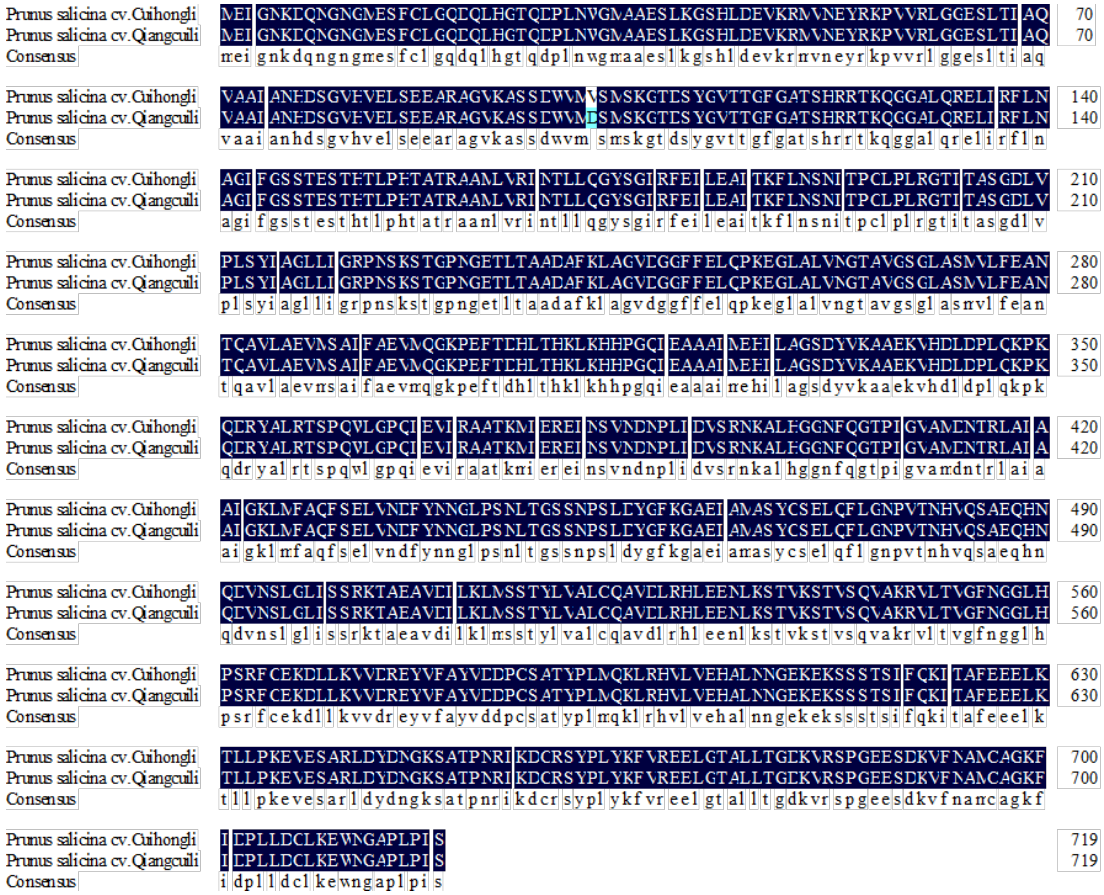

Fig. 2 Alignment of predicted amino acid sequence of PAL of 'Cuihongli' and 'Qiangcuili'

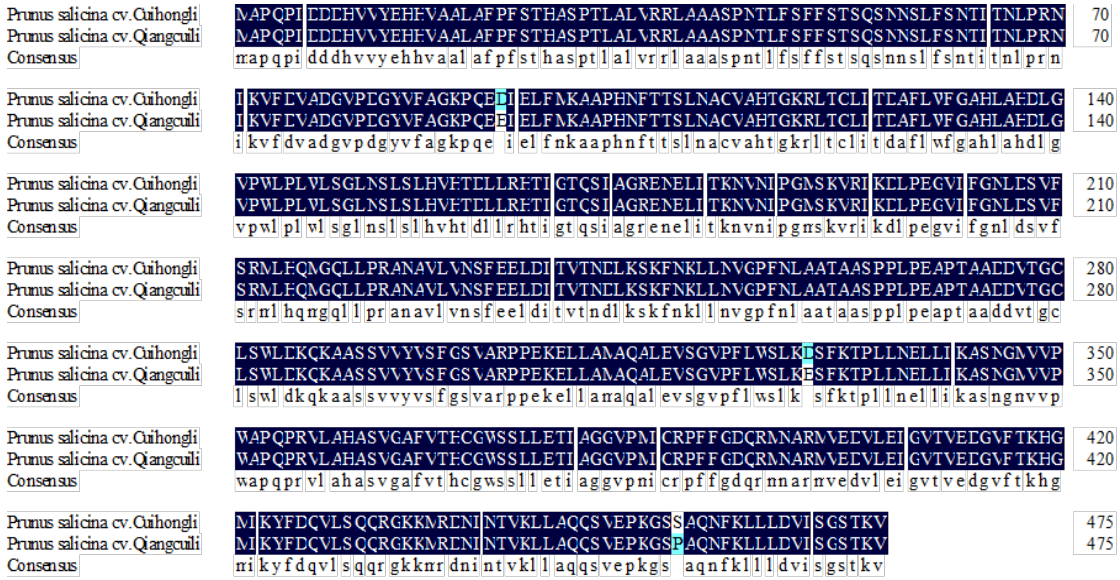

Fig. 3 Alignment of predicted amino acid sequence of UFGT of 'Cuihongli' and 'Qiangcuili'

3.3. Amino acid sequence alignment and phylogenetic tree construction of MYB10 in 'Cuihongli'.

The obtained MYB10 amino acid sequence was analyzed and searched on NCBI. Results showed that the amino acid sequence of 'Cuihongli' was similar to that of Prunus cerasifera, Prunus armeniaca, Prunus avium, Prunus persica: 99.6%, 97.5%, 94.2% and 93.3%, respectively(Table 2). The phylogenetic tree of 'Cuihongli' MYB10 gene and other plants was constructed by mega5.10 software (Fig. 4). The genetic relationship between Chinese plum and other MYB10 protein was further analyzed. From the evolutionary tree map, it showed that the genetic distance between Chinese plum and Prunus cerasifera, Prunus armeniaca, Prunus avium, Prunus persica of Rosaceae was close, which was basically consistent with homology analysis.

Table 2 Comparison of MYB10 Amino Acid sequences homology between 'Cuihongli' and different plants

Species	Gene Bank number	Homology（%）
Litchi chinensis	APP94121.1	51.7
Arabidopsis thaliana	AAS10033.1	45.3
Eriobotrya japonica	ABX71484.1	71.0
Malus domestica	ABK58138.1	71.7
Vitis vinifera	ABB87010.1	48.9
Prunus persica	AKI23599.1	93.3
Citrus sinensis	NP_001275818.1	52.3
Prunus avium	ABX71493.1	94.2
Pyrus communis	ABX71487.1	72.2
Prunus armeniaca	ABX71490.1	97.5
Prunus cerasifera	AKV89248.1	99.6

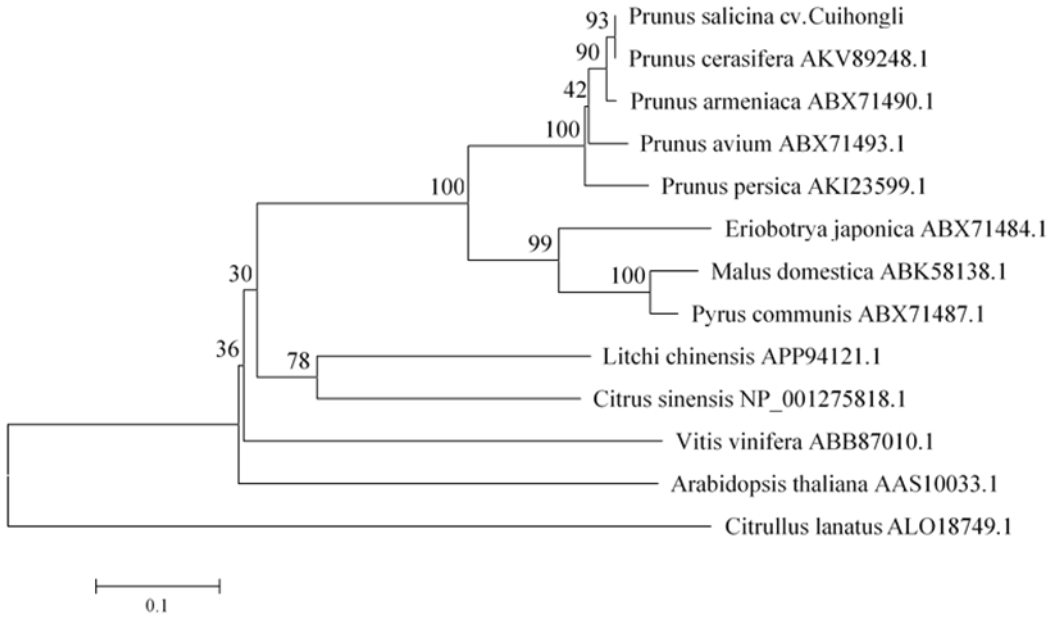

Fig.4 Phylogenetic tree of MYB10 homologous sequence

3.4. Amino acid sequence alignment and phylogenetic tree construction of PAL in 'Cuihongli' and 'Qiangcuili'

The amino acid sequence of PAL was analyzed and searched on NCBI. The results showed that the amino acid sequence of 'Cuihongli' was 99.0%, 98.6% and 98.5% similar to that of Prunus armeniaca, Prunus mume and Prunus persica, respectively. The similarity of the amino acid sequence between 'Qiangcuili' with Prunus armeniaca, Prunus mume and Prunus persica was 99.2%, 98.7% and 98.6%, respectively. The similarity between the two sequences and Arabidopsis thaliana was 80.6%(Table 3). The phylogenetic trees of 'Cuihongli' and 'Qiangcuili' PAL proteins with other plants were constructed by MEGA5.10 software (Fig. 5). The genetic relationship between Chinese plum and other plant PAL proteins was further analyzed. From the evolutionary tree map, it showed that the genetic distance between Chinese plum and Prunus armeniaca, Prunus mume and Prunus persica, which were also belong to Rosaceae, was close, while the genetic distance between Chinese plum and Arabidopsis thaliana is the furthest, which was consistent with homology analysis.

Table 3 The homology comparison of amino acid sequences of PAL between 'Cuihongli', 'Qiangcuili' and other plants

Species	Gene Bank number	Homology（%）	
		Prunus. *Salicina* cv.Cuihongli	*Prunus salicina* cv.Qiangcuili
Castanea mollissima	APF46971.1	86.4	86.6
Ziziphus jujuba	XP_015877186.1	85.4	85.4
Juglans regia	XP_018828772.1	85.7	85.7
Daucus carota	BAC56977.1	84.7	84.7

Mangifera indica	AIY24976.1	85.7	85.7
Populus tomentosa	AFZ78651.1	85.5	85.6
Prunus mume	XP_008233304.1	98.6	98.7
Arabidopsis thaliana	AAC18871.1	80.6	80.6
Vitis vinifera	ABM67591.1	85.2	85.2
Malus domestica	XP_008366650.1	91.2	91.4
Prunus persica	XP_007220630.2	98.5	98.6
Prunus armeniaca	AOC97438.1	99.0	99.2

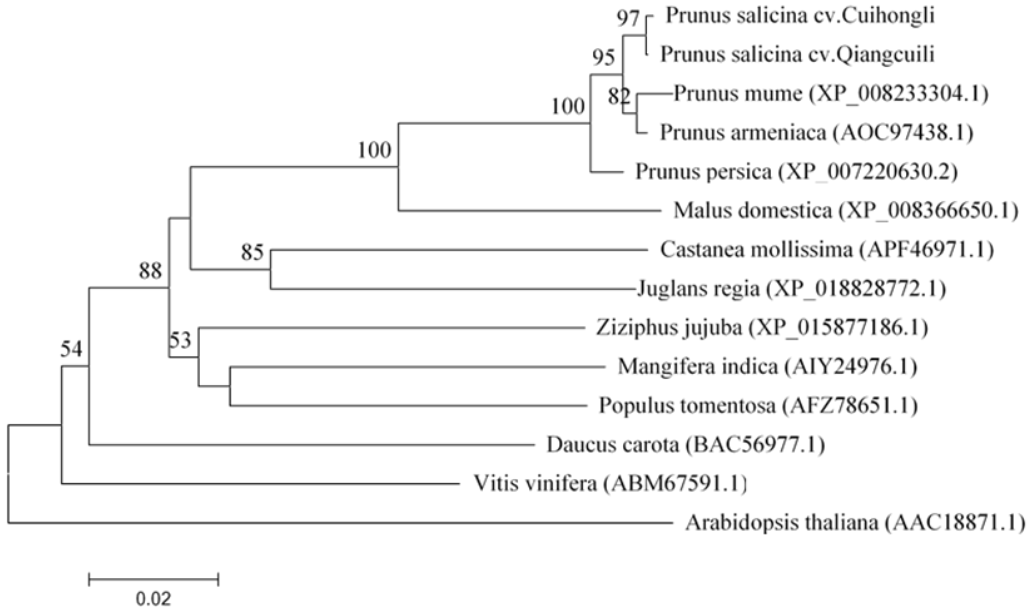

Fig. 5 The phylogenetic tree of PAL homology sequence

3.5. Amino acid sequence alignment and phylogenetic tree construction of UFGT in 'Cuihongli' and 'Qiangcuili'

The amino acid sequence of UFGT was analyzed and searched on NCBI. The results showed that the amino acid sequence of 'Cuihongli' was similar to that of Prunus cerasifera, Prunus mume, Prunus avium and Prunus persica, which were 98.3%, 97.7%, 97.0% and 96.2%, respectively. The similarity with Litchi chinensis, Actinidia chinensis and Arabidopsis thaliana was 55.4%, 54.0% and 53.1%, respectively. The similarity of the amino acid sequence between 'Qiangcuili' with Prunus cerasifera, Prunus mume and Prunus avium was 97.7%, 97.0% and 96.4%, respectively. the similarity with Litchi chinensis, Actinidia chinensis and Arabidopsis thaliana was 55.0%, 53.3% and 53.1%, respectively (Table 4). The phylogenetic trees of 'Cuihongli' and 'Qiangcuili' UFGT proteins with other plants were constructed by MEGA5.10 software (Fig. 6). The genetic relationship between Chinese plum and other plant UFGT proteins was further analyzed. From the evolutionary tree map, it showed that the genetic distance between Chinese plum and Prunus cerasifera, Prunus mume, Prunus avium and Prunus persica of Rosaceae was close, which was basically consistent with homology analysis.

Table 4 The homology comparison of amino acid sequences of UFGT between 'Cuihongli', 'Qiangcuili' and other plants

Species	Gene Bank number	Homology（%）	
		Prunus. *Salicina* cv.Cuihongli	*Prunus*. *Salicina* cv.Qiangcuili
Fragaria ananassa	AAU12366.1	63.2	62.5
Corchorus capsularis	OMO98378.1	59.2	59.0
Litchi chinensis	ALH45553.1	55.4	55.0
Prunus mume	XP_008234582.1	97.7	97.0
Arabidopsis thaliana	AAL61932.1	53.1	53.1
Malus domestica	BAI44431.1	68.9	68.2
Vitis vinifera	BAB41020.1	60.0	59.4
Morus alba	AOV62766.1	59.1	59.1
Prunus persica	AFP90753.1	96.2	95.6

Prunus avium	AHL45018.1	97.0	96.4
Pyrus communis	AGL81353.1	74.1	73.4
Prunus cerasifera	AKV89253.1	98.3	97.7
Actinidia chinensis	ADC34700.1	54.0	53.3

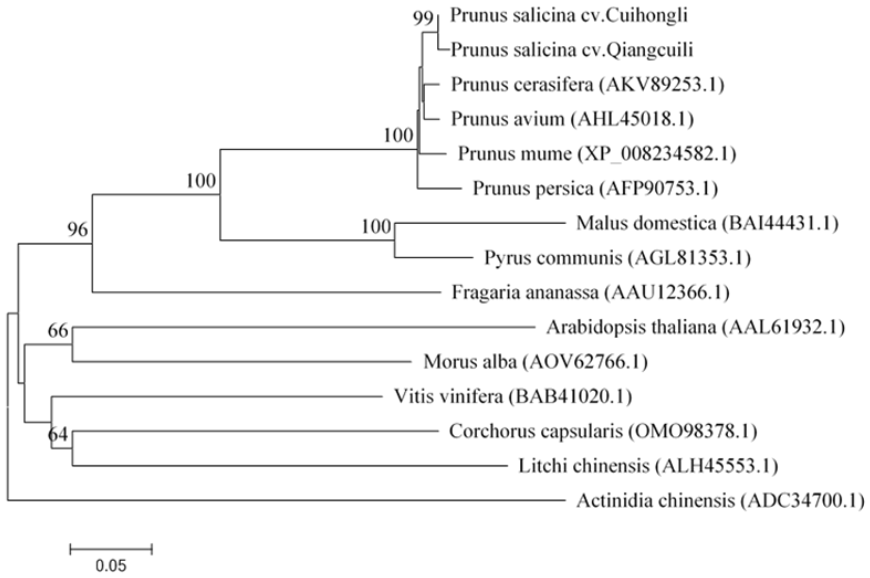

Fig. 6 The phylogenetic tree of UFGT homology sequence

4. Conclusions

Anthocyanin is widely distributed in plants. At present, anthocyanin synthesis related genes have been cloned in mango[14], tulip[15], peach[16] and many other plants. Much researches found transcription factors regulating anthocyanin accumulation in fruits, among which *MYB10* was a hot topic, but it was rarely reported on plum, especially on 'Cuihongli'. In this experiment, the sequences of the structural genes *PAL*, *UFGT* and the regulatory gene *MYB10* in 'Cuihongli' and 'Qiangcuili' were obtained by homologous sequence cloning. Among them, the full length of *PAL* was 2160 bp, encoding 719 amino acids; the full length of *UFGT* was 1428 bp, encoding 475 amino acids. Comparing the amino acid sequences of the two genes in the two varieties, the differences were one amino acid between PAL and three amino acids between UFGT. The phylogenetic tree of UFGT encoding protein found that the relationship between Chinese plum and *Prunus cerasifera*, *Prunus mume*, *Prunus avium* and *Prunus persica* was close. The CDS region of *MYB10* gene was 732 bp, and the total length of MYB10 protein was 243 aa. Through the analysis of the conserved domain of 'Cuihongli' MYB10 protein, we found that it belongs to the SANT superfamily and contained many active sites, which was consistent with the analysis of *MYB* gene sequence of *Pyrus betulaefolia* by Ran Kun *et al*. [17]. In the experiment, the target band of *MYB10* was not successfully obtained from 'Qiangcuili'. It was speculated that the expression of *MYB10* in 'Qiangcuili' was extremely low, resulting in no accumulation of anthocyanins. The experimental results provided a theoretical basis for the study of the coloring difference between 'Cuihongli' and 'Qiangcuili' in the future.

Acknowledgments

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