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 synthesizes 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 in 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 showed 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 °C 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.

| Primer   | Nucleotide of sequences (5′-3′)                        |
|----------|------------------------------------------------------|
| kMYBI0F  | ATGGAGGGWTATAACTTGGGTGTGAGAAAAGGAGC                 |
| kMYBI0R  | CTATTCTCTTTGGAATTGATTCCAAAAGGTCGCCACGC              |
| PsPALF   | ATGGAAATCGGCAATAAGG                                  |
| PsPALR   | GCSATAACCAGCCTCTCTA                                  |
| PsUFGTF  | TATATGGCACCRCACCGAT                                  |
| PsUFGTR  | AAGTACAGCTCGGTTATCT                                 |

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.
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).
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.
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| 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         |

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.

| Species              | Gene Bank number | Homology (%) |
|----------------------|------------------|--------------|
| Castanea mollissima  | APF46971.1       | 86.4         |
| Ziziphus jujuba      | XP_015877186.1   | 85.4         |
| Juglans regia        | XP_018828772.1   | 85.7         |
| Daucus carota        | BAC56977.1       | 84.7         |

| Species              | Gene Bank number | Homology (%) |
|----------------------|------------------|--------------|
| Prunus, Salicina     |                  |              |
| cv. Cuihongli        |                  |              |
| Prunus salicina      |                  |              |
| cv. Qiangcuili       |                  |              |
| Castanea mollissima  | APF46971.1       | 86.4         |
| Ziziphus jujuba      | XP_015877186.1   | 85.4         |
| Juglans regia        | XP_018828772.1   | 85.7         |
| Daucus carota        | BAC56977.1       | 84.7         |
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 other plant UFGT proteins was further analyzed. From the evolutionary tree map, it showed that the genetic distance 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 other plant UFGT proteins was further analyzed. From the evolutionary tree map, it showed that the genetic distance 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 other plant UFGT proteins was further analyzed.
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.

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