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Cloning and Sequence Analysis of a MYB Transcription Factor (MYB44) in *Prunus persica*

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Abstract

Anthocyanins are biosynthesized through a series of enzymes with the transcript levels of their encoding genes commonly regulated by the MBW transcription complex containing MYB, bHLH and WD40 transcription factors. As one of the largest plant transcription factor families, MYB (v-myb avian myeloblastosis viral oncogene homolog) transcription factors play an important role in plant anthocyanin biosynthetic pathway. In order to investigate the relationship MYB transcription factors of peach peel, a transcription factor gene MYB44 were isolated from *Prunus persica* by using RT-PCR technology. Sequence analysis indicated MYB44 that were 669bp, encoding 223 amino acids, with a calculated molecular mass of 25.68kDa and an isoelectric point of 9.06, which was designated as *PpMYB44* (GenBank accession No. KX852400). Bioinformatics analysis showed that the deduced PpMYB44, which shared more than 60% identities with the MYB protein from other plants. The protein predication of PpMYB44 showed that there were a function domains MYB_DNA-binding and typical structure of SANT superfamily. Predicted protein secondary structure showed that PpMYB44 protein contents of the structure with alpha helix (Hh) 35.59%, extended strand (Ee) 19.82%, random coil (Cc) 35.58%, and beta turn (Tt) 9.01%, it had high similarities with Malus domestica, *Pyrus×bretschneideri,* and *Medicago truncatula* MYB proteins. Phylogenetic analysis results revealed that PpMYB44 were closely related to anthocyanin biosynthesis MYB transcription factors in *Prunus mume*, *Malus domestica*, and *Pyrus×bretschneideri*. These results indicate that PpMYB44 belongs to MYB families and responsible for anthocyanin biosynthetic in peach peels.

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Keywords

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Introduction

Prunus persica is one species pertaining to the *Prunus* genus which containing other economically significant species. Peach is a member of the Rosaceae, which ranks as the third most agronomically important plant family in temperate regions (Dirlewanger et al., 2002). Fruit producers must satisfy consumers by producing fruits of good flavour, colour and texture and must also provide marketers with fruits resistant to mechanical damage. In peach production, peel pigmentation improvement has been much slower to respond because this character is complex and difficult to control (Iglesias and Echeverría, 2009). The biological and genetic bases of fruit quality are still poorly known. Researches shown that anthocyanin content and

composition are major determinants of peach quality (Bassi and Selli, 1990).

Anthocyanidin, a flavonoid, is a natural pigment widely found in plants. It usually binds to glucose and other sugars to form glycoside called anthocyanin. Anthocyanin is an important secondary metabolite in plants and mostly distributed in the vacuoles of flowers, fruits, seeds, leaves, and other plant organs, giving plants all shades of colors from orange red to purple blue(Shimada et al., 2005; Wellmann et al., 2006).

Anthocyanins are the principal pigment in peels with important nutritional and medical usages. Large amount of anthocyanin deposits in mature fruits, which not only gives fruits rich colors to attract consumers, but also can prevent the occurrences of cardiovascular disease, neurons and aging-related diseases, cancers, and cerebral central embolism and ischemia, improve glucose homeostasis, and enhance visual acuity, it becomes one of the major measures of plant nutrients and food health. For plants themselves. Anthocyanin works to resist low temperature, drought, and fungal infections, to defense damages caused by UV and pests, to attract pollinators, and to facilitate seed dispersal (Gonzalez et al., 2008; Castellarin et al., 2007).

The research on biosynthesis of anthocyanins has been focused on the transcriptional regulation of structural genes in latest years. The transcriptional factors involved in biosynthesis of anthocyanins mainly include three kinds of protein families MYB, bHLH, and WD40.The interactions of different transcription factors are critical in gene regulation in various cellular metabolisms. Temporally and spatially coordinated expressions of anthocyanin biosynthesis-related structural genes are found in the process of plant development, which is caused by the co-regulation of transcriptional genes (Hichri et al., 2011).

MYB gene, as a regulatory gene in anthocyanin synthesis, encodes a transcription factor. This transcription factor can bind to proteins bHLH and WD40 to form a complex, which can co-regulate the expression intensity and pattern of structural genes in the anthocyanin synthesis pathway (Allan et al., 2008), so as to control the temporal and spatial changes of anthocyanin synthesis (Fig. 1). Scientific research workers have cloned the MYB gene sequence from all kinds of plants, such as *Arabidopsis thaliana* (Zhang et al., 2003), *Petunia hybrida* Vilm. (Quattrocchio et al., 1999), *Perilla frutescens* (L.) Britt (Sompornpailin et al., 2002), *Garcinia mangostana* L. (Palapol et al., 2009), *Fragaria×ananassa* (Wang et al., 2010), *Pyrus* spp. (Feng et al., 2010), *Citrus sinensis* (Butelli et al., 2012), *Vitis vinifera* (Kobayashi et al., 2002), *Myrica rubra* (Feng et al., 2012), and *Zea mays* (Paz-Ares et al., 1987). But so far, there has not been a report on the complete MYB44 sequence and expression analysis of peach peel. In this experiment, with peach peel as subject, cloned MYB44 gene fragment by applying homology cloning and conducted bioinformatics analysis, aiming at laying the foundation for the full-length clone of the MYB44 gene, structural-functional analysis and the role of this gene in the anthocyanin biosynthetic pathway in peach peel.

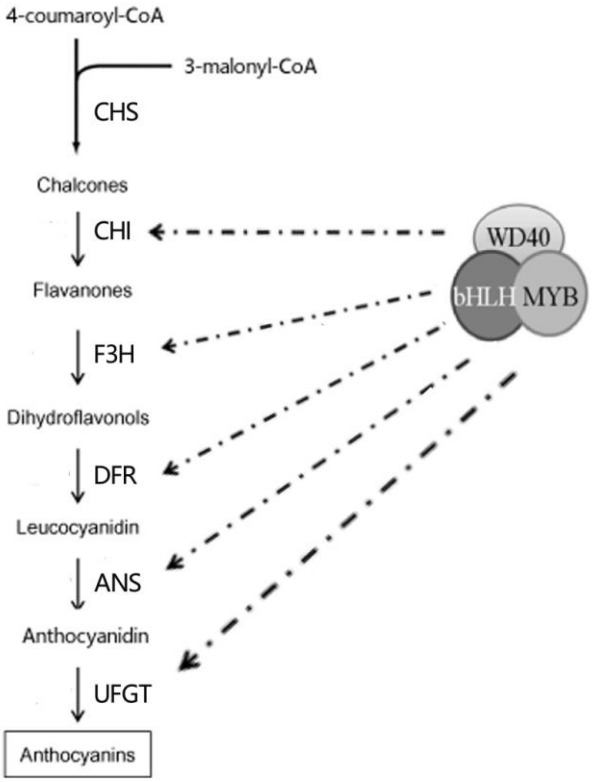

Fig. 1: Transcriptional regulation of anthocyanin biosynthesis in *Prunus persica* by MYB transcription factor. CHS：Chalcone synthase; CHI: Chalcone isomerase; F3H: Flavanone 3-hydroxylase; DFR: Dihydroflavonol 4-reductase; ANS: Anthocyanidin synthase; UFGT: UDP g1ucose-Flavonoid 3-*O*-glucosyltransferase.

Materials and methods

Plant materials

Six-year-old grafts of peach were grown on the deciduous fruit tree base in the horticultural farm of Yangtze University, China. DNA and RNA extraction

were collected from peach peels, which were immediately frozen in liquid nitrogen and kept at −80°C (DW-86L626, Haier Co. Ltd., China) until analyses.

RNA extraction and cDNA synthesis

Total RNA was extracted from the peels of peach tissues based on the MiniBEST Plant RNA Extraction kit (TaKaRa, China). Peels of peach weighing 5g were immediately ground to powder in liquid nitrogen, filtered and after that centrifuged at 12, 000 × g for 5 min under the condition of 4°C.The concentration and quality of the total RNA were tested by absorbance ratio of OD260/280, spectrophotometer and 1 % (w/v) agarose gel electrophoresis, respectively. First-strand cDNA was synthesised with using cDNA Prime Script® 1st Strand cDNA Synthesis Kit (TaKaRa, China). Reaction system contained Oligo dT Primer 2µl, dNTP mixture 2µl, Total RNA 16µl; 5×Primscript buffer 8µl, RNase inhibitor 1µl, Prime script RTase 2µl, RNA free H_2O 9µl The reaction steps were 45°C for 30 min, followed by 75°C for 20 min.

Isolation of *PpMYB44*

A genomic fragment of *PpMYB44* was amplified using the MYB44-FP (5′- ATGTTTTCTGTTCCATTTTTTTCT-3′) and MYB44-RP (5′- TCAAAAACCAAAGATGCACG-3′) specific primers were designed and synthesized (Shanghai Sangon, China) based on the transcriptome sequencing data of peach. The PCR reaction conditions was performed at 96°C for 3 min, and then subjected to 31 cycles of 95°C for 15 s, 52°C for 40 s and 72°C for 45 s, and then extension at 72°C for 5 min, final four degrees heat preservation. The obtained amplified were separated in 1.5% gel electrophoresis and purified by a AxyPrep DNA Gel Extraction Kit, and then the purified product was cloned into the pMD20-T vector (TaKaRa, China), after that sequenced.

Bioinformatics analysis

The obtained nucleotide sequence and deduced amino acid sequence were compared through database search using online bioinformatics tools (NCBI, http://www.ncbi.nlm.nih.gov). The software DNAman 6.0 was used to analyze *PpMYB44* transcription factor gene sequence and amino acid composition. *PpMYB44* and other MYB proteins obtained from GenBank were aligned with the software Vector NTI suit 10.0 program. The BioEdit 6.0 and MEGA 6.1 were used to construct the phylogenetic tree by Neighbour-Joining method and multiple alignment analysis of MYB44 amino acid sequences. The bootstrap statistical analysis was carried out with 1000 replicates. Online tool of ExPASy-ProtParam tool (www.expasy.org/tools/protparam.html) was used to deduce protein molecular weight and iso-electric point.

Results

cDNA cloning of *PpMYB44*

Based on the sequences of EST, the cDNA sequence of *PpMYB44* was obtained according to the primer pair MYB44-FP and MYB44-RP and total RNA reverse transcription product. A 700 bp product was obtained and sequenced (Fig. 2). The cloned full-length open reading frame (ORF) of the *PpMYB44* was 669 bp long encodes 223 amino acids (Fig. 3), after removal of the primer. The cDNA sequence of *PpMYB44* had high similarity with other MYB genes, and the G+C content was 25%.

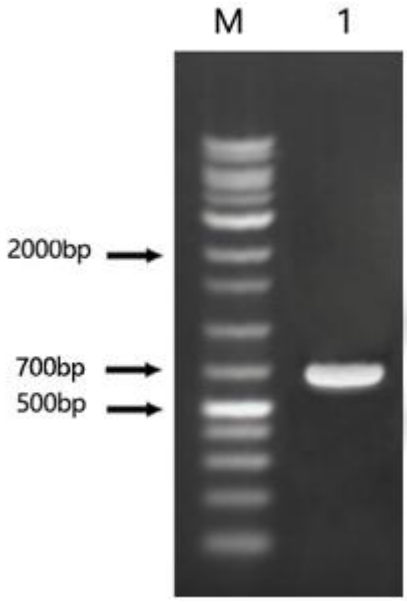

Fig. 2: Gel electrophoresis of *PpMYB44* gene. Note: M: DNA marker, 1: cDNA of *PpMYB44*.

Characterization of the deduced PpMYB44 protein

Through analysis with the Computer pI/Mw, we found predicted PpMYB44 amino acid calculated molecular mass of 25.68kDa and an isoelectric point of 9.06. Blastp aligning analysis found the protein predication of PpMYB44 showed that there were a function domains MYB_DNA-binding and typical structure of SANT superfamily; Multi-domains are domain models that

contain SANT, speE, PLN03212, and REB1 domains (Fig. 4). The secondary structures of PpMYB44 proteins were predicted by using SOPMA tool. It was found that PpMYB44 contents the structure with alpha helix (Hh) 35.59%, extended strand (Ee) 19.82%, random coil (Cc) 35.58%, and beta turn (Tt) 9.01% (Fig. 5). The secondary structures of deduced PpMYB44 had high similarities with *Malus domestica*, *Pyrus*×*bretschneideri*, and *Medicago truncatula* MYB proteins (Table 1).

```
1      ATGTTTTCTGTTCCATTTTTTTCTTCTCTCTTTTGCTTTTTAGTGTTTATTCTGATCTTT
1       M  F  S  V  P  F  F  S  S  L  F  C  F  L  V  F  I  L  I  F
61     AACACATCTCTCTGTGTTTGTCTATGTTCAAAAGGTAAGAGCTGTAGACTAAGATGGTTT
21      N  T  S  L  C  V  C  L  C  S  K  G  K  S  C  R  L  R  W  F
121    AACCAGTTGGACCCAAGGATCAACAGAAGAGCTTTTACTGAAGAAGAAGAAGATAGACTA
41      N  Q  L  D  P  R  I  N  R  R  A  F  T  E  E  E  E  D  R  L
181    ATGCAAGCTCATAGAGTATATGGAAACAAATGGGCCATGATAGCTAGGCTCTTTCCTGGA
61      M  Q  A  H  R  V  Y  G  N  K  W  A  M  I  A  R  L  F  P  G
241    AGAACTGATAATGCTGTCAAGAACCATTGGCATGTTATAATGGCCAGGAAGTACAGAGAA
81      R  T  D  N  A  V  K  N  H  W  H  V  I  M  A  R  K  Y  R  E
301    CAATCCAGTGCTTACAGGAGGAGGAAGCTCAGTCAAACTGTTTACAGAAGAATGGAGGAA
101     Q  S  S  A  Y  R  R  R  K  L  S  Q  T  V  Y  R  R  M  E  E
361    GATCCAAGCTTCAATGTCTCAAGGAGTACAGAGCCTCCATCTTATTGTAGTCTCAATCTC
121     D  P  S  F  N  V  S  R  S  T  E  P  P  S  Y  C  S  L  N  L
421    CCCCACAATGGAGGTCTCAGCAATAGCACTTTATCTCCTTTCTCATATGGAACAAGCTAT
141     P  H  N  G  G  L  S  N  S  T  L  S  P  F  S  Y  G  T  S  Y
481    AATGGTGTTGTTGGTTGGGGGGTTGACTATTATGGCTCAAATGGCTCACCCAACATGACC
161     N  G  V  V  G  W  G  V  D  Y  Y  G  S  N  G  S  P  N  M  T
541    AGTGCGGAAGAAGAAGAAACAATCCCAAGCAAAAAAGTCCTTCCTTTCGAATTCTTCCCC
181     S  A  E  E  E  E  T  I  P  S  K  K  V  L  P  F  E  F  F  P
601    GGTATGTCATTCAGGATCCCTCATTTCCTTTCTCACGATTCTTTCTGTTCGTGCATCTTT
201     G  M  S  F  R  I  P  H  F  L  S  H  D  S  F  C  S  C  I  F
661    GGTTTTTGA
221     G  F  *
```

Fig. 3: Nucleotide sequence and deduced amino acid sequence of *PpMYB44*.

Table 1. Secondary structure of MYB proteins (unit: %).

Species	Alpha helix (Hh)	Extended strand (Ee)	Random coil (Cc)	Beta turn (Tt)
Prunus persica	35.59%	19.82%	35.58%	9.01%
Malus domestica	32.50%	16.11%	40.83%	10.56%
Pyrus×*bretschneideri*	31.02%	15.79%	42.94%	10.25%
Medicago truncatula	30.44%	17.33%	44.03%	8.20%

Multiple alignments of PpMYB44 protein

Sequence comparison by performing Blast P Search (http://www.ncbi.nih.gov) showed that PpMYB44 had higher homology with other MYB, the amino acid sequences multialignment (Fig. 6) showed that PpMYB44 had high identities with *Jatropha curcas* (94% identities, AIT52213.1); *Cucumis sativus* (91% identities, KGN51930.1); *Salvia miltiorrhiza* (89% identities, AGN52127.1); *Erythranthe lewisii* (81% identities, ALE33742.1); *Malus domestica* (74% identities, ADL36763.1); *Gossypium arboreum* (66% identities, KHG19063.1); *Medicago truncatula* (63% identities, AES96795.1); *Cajanus cajan* (62% identities, KYP76593.1); *Glycine max* (62% identities, KRH50566.1); *Glycine soja* (60% identities, KHN42915.1). The homology of MYB gene sequence among different species suggested that MYB was strongly conserved during molecular evolution, mentioned above indicated that PpMYB44 was a member of the MYB family.

Molecular evolution analysis of PpMYB44

To further explore the evolutionary relationships among PpMYB44 and other proteins referred to the anthocyanidin biosynthesis in plants, a phylogenetic tree was constructed applied to the MEGA 6.1 software tools. As shown in Fig. 7, phylogenetic analysis showed that PpMYB44 have a closer relationship to *Prunus mume* MYB protein than to other MYB proteins. The PpMYB44 protein together with *Prunus mume*, *Malus domestica*, *Pyrus*×*bretschneideri*, *Medicago truncatula*, and *Trifolium subterraneum* were grouped into a functional cluster. Based on this result, it can be suggested that PpMYB44 belonged to MYB gene family, PpMYB44 may have similar functions with other MYB proteins.

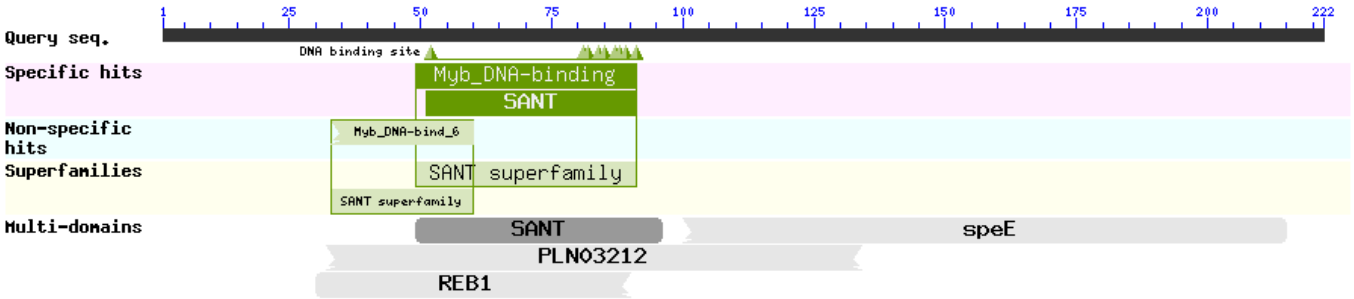

Fig. 4: The amino acid conservative sequence analysis of PpMYB44. Non-specific hits is the most conserved domain model matching with the query sequence; A superfamily cluster is a set of conserved domain models that generate overlapping annotation on the same protein sequences; Multi-domains are domain models that contain multiple single domains.

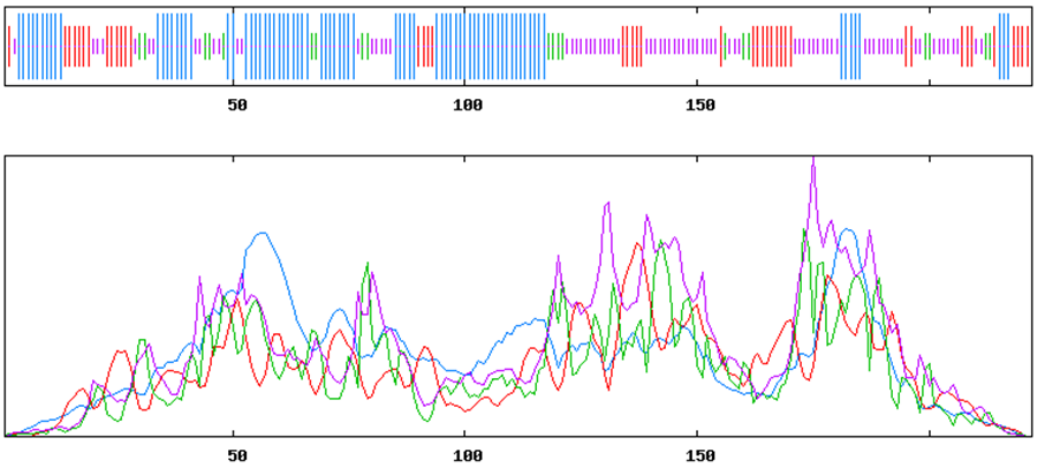

Fig. 5: The predicted secondary structures of PpMYB44 protein.

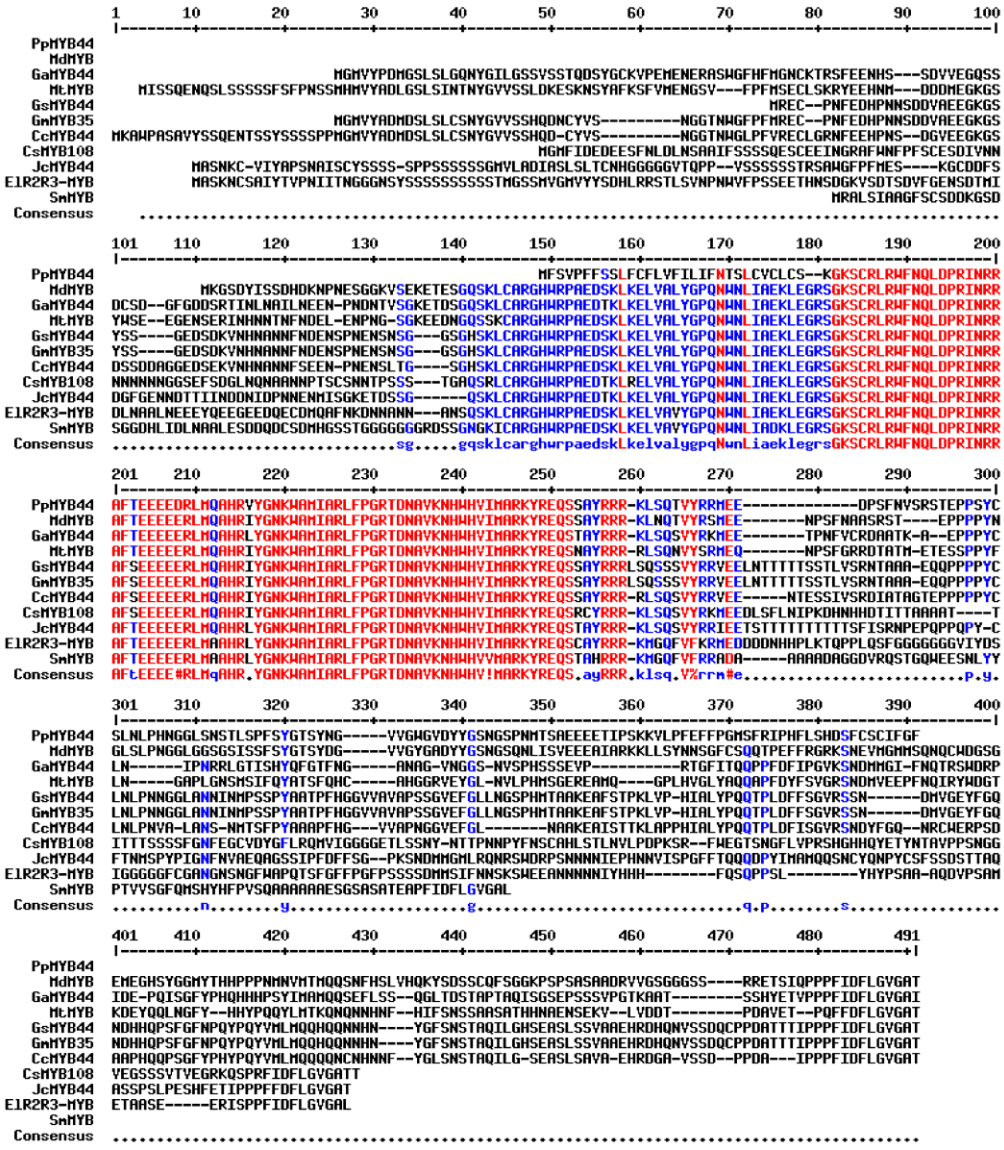

Fig. 6: Sequence multi-alignment of the deduced PpMYB44 protein with other MYB proteins. The accession numbers of MYB proteins and translation of their names are shown as follows, PpMYB44: *Prunus persica*; MdMYB: *Malus domestica* (ADL36763.1); GaMYB44: *Gossypium arboretum* (KHG 19063.1); GsMYB44: *Glycine soja* (KHN42915.1); GmMYB35: *Glycine max* (KRH50566.1); MtMYB: *Medicago truncatula* (AES96795.1); CcMYB44: *Cajanus cajan* (KYP76593.1); CsMYB108: *Cucumis sativus* (KGN51930.1); JcMYB44: *Jatropha curcas* (AIT52213.1); ElR2R3-MYB: *Erythranthe lewisii* (ALE33742.1); SmMYB: *Salvia miltiorrhiza* (AGN 52127.1). High consensus is indicated in red colour (default: 90%); low consensus is indicated in blue colour (default: 50%); non-similar amino acids are indicated with black colour; white is the background colour.

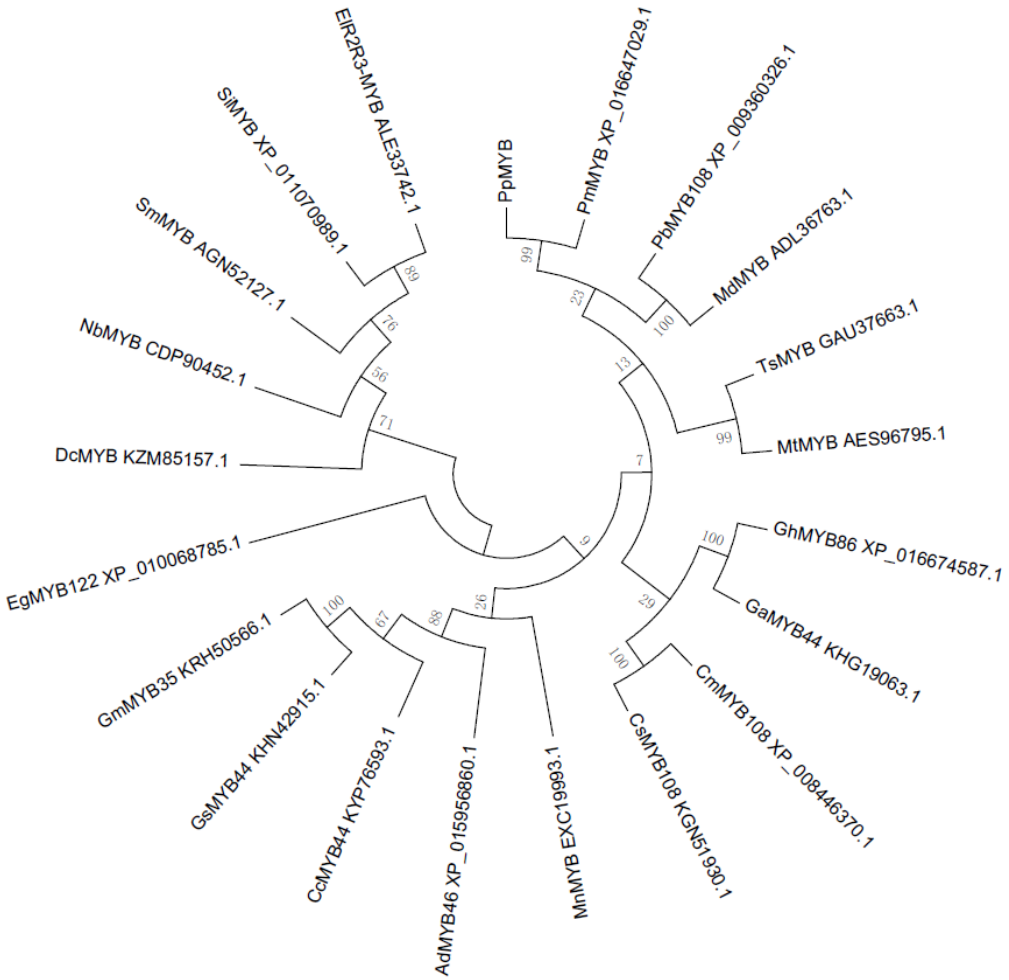

Fig. 7: Molecular phylogenetic tree of the deduced amino acid sequences of MYBs from plants. The numbers on the branches represent bootstrap support for 1,000 replicates. The MYBs used in phylogenetic tree analysis were from plants including PpMYB44: *Prunus persica*; MdMYB: *Malus domestica* (ADL36763.1); GaMYB44: *Gossypium arboreum* (KHG19063.1); GsMYB44: *Glycine soja* (KHN42915.1); GmMYB35: *Glycine max* (KRH50566.1); MtMYB: *Medicago truncatula* (AES96795.1); CcMYB44: *Cajanus cajan* (KYP76593.1); CsMYB108: *Cucumis sativus* (KGN51930.1); ElR2R3-MYB: *Erythranthe lewisii* (ALE33742.1); SmMYB: *Salvia miltiorrhiza* (AGN52127.1): PmMYB: *Prunus mume* (XP_016647029.1); PbMYB108: *Pyrus×bretschneideri* (XP_009360326.1); GhMYB86*: Gossypium hirsutum* (XP_016674587.1); TsMYB: *Trifolium subterraneum* (GAU37663.1); CmMYB108: *Cucumis melo* (XP_008446370.1); NbMYB: *Nicotiana benthamiana* (CDP90452.1); MnMYB: *Morus notabilis* (EXC19993.1); SiMYB: *Sesamum indicum* (XP_011070989.1); AdMYB46: *Arachis duranensis* (XP_015956860.1); EgMYB122: *Eucalyptus grandis* (XP_010068785.1); DcMYB: *Daucus carota* subsp. sativus (KZM85157.1).

Discussion

This study isolated a MYB gene (*PpMYB44*) from peach fruit peel by using RT-PCR technique. Results show that the full-length ORF of *PpMYB44* was 669 bp encoding 223 amino acids with a calculated molecular mass of 25.68kDa and an isoelectric point of 9.06. Blastp aligning analysis found the protein predication of PpMYB44 showed that there were a function domains MYB_DNA-binding and typical structure of SANT superfamily; Multi-domains are domain models that contain SANT, speE, PLN03212, and REB1 domains. In all these MYB protein containing *Malus domestica*, *Pyrus×bretschneideri*, and *Medicago truncatula*, evident signal peptide, transmembrane region and coiled—coil domain were predicted, and similar ratio in secondary structure composition made from Alpha, Extended strand, and Random coil were detected. The results of nucleic acid homology alignment revealed that the homology of MYB in *Prunus persica* was very high with other plants. It was found that the PpMYB44 gene encoding for the protein had close relationship with *Prunus mume*, *Malus*

domestica, *Pyrus×bretschneideri*, *Medicago truncatula*, and *Trifolium subterraneum* through phylogenetic analysis. The above results show that the PpMYB44 protein belonged to MYB proteins family.

Interactions of several structural genes, regulatory genes, and environmental factors are involved in the biosynthesis of anthocyanin. Studies have demonstrated that the biosynthesis of anthocyanin in grapes showed significant differences with the development of fruits, and its regulation mechanism involves the synergistic effects of transcription factors MYB, bHLH, and WD40 (Hichri et al., 2010). MYB transcription factor specifically regulates the biosynthesis of anthocyanin in apple skin, and its transcript level is strongly induced by light and positively correlated to the accumulation of anthocyanin (Takos et al., 2006). Scientific research showed that the expressions of anthocyanin synthesis genes are up-regulated during the ripening period of red bayberry fruits, combined with the enhanced expression of the MYB transcription factor that specifically regulates the biosynthesis of anthocyanin. The over expression of the MYB transcription factor in Arabidopsis can enhance the accumulation of anthocyanin in the whole plant (Niu et al., 2010; Feng et al., 2012). In recent years, an increasing number of studies have indicated that there is certain synergistic effect between the expression intensities of the structural genes of anthocyanin synthesis, which is co-regulated by the transcription complex formed by transcription factors MYB, bHLH, and WD40.

MYB transcription factor is one of the largest families of transcription factors in plants. Although there are certain conservations in sequences and similarities in structures, functions of MYB protein are of significant differences among different species, different individual plant, and even different organs in the same plant (Liu et al., 2004; Singh et al., 2002; Wilkins et al., 2009). At present, functions of many MYB transcription factors remain unclear, so it is of essential importance to clone MYB genes and to clarify their functions in plant, especially in the respect of secondary metabolism regulation and responses to hormones and environmental stress. This study only cloned gene fragments of the MYB from peach peel; the cloning of the full-length sequence of the UFGT from the torch pear should be further carried out to study the expression level and pattern of this gene in each developmental stage. According to the cloned *PpMYB44* gene sequence, this study will subsequently construct a plant expression vector of the *PpMYB44* gene, aiming to further verifying the function of the *PpMYB44* gene in anthocyanin metabolism and increase the content of anthocyanin in peach fruits by genetic engineering method.

Conclusion

The full-length ORF of *PpMYB44* was 669 bp encoding 223 amino acids with a calculated molecular mass of 25.68kDa and an isoelectric point of 9.06 was isolated by RT-PCR through *Prunus persica* peels. The protein predication of PpMYB44 showed that there were a function domains MYB_DNA-binding and typical structure of SANT superfamily. Predicted protein secondary structure showed that PpMYB44 protein had high similarities with other plants of MYB proteins. Phylogenetic analysis results revealed that PpMYB44 were closely related to anthocyanin biosynthesis MYB transcription factors in *Prunus mume*, *Malus domestica*, and *Pyrus×bretschneideri*. These results indicate that PpMYB44 belongs to MYB families and responsible for anthocyanin synthesis in peach fruit peel.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

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