Research Article

Functional Analysis of a Pomegranate (*Punica granatum* L.) MYB Transcription Factor Involved in the Regulation of Anthocyanin Biosynthesis

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Background: Pomegranate fruit (*Punica granatum* L.) is a rich source of anthocyanin pigments resulting in vibrant colours and anti-oxidant contents. Although the intensity and pattern of anthocyanin biosynthesis in fruit are strongly influenced by R2R3-MYB transcription factors, little is known about the regulation and role of MYB in anthocyanin pathway of pomegranate.
Objectives: The present study was conducted to elucidate the relationship between the expression of MYB transcription factor and the anthocyanin accumulation during the colour development phase of pomegranate fruits.
Materials and Methods: In this work, R2R3-MYB transcription factor (PgMYB) was isolated and characterized from pomegranate skin through RACE-PCR. The expression of *PgMYB* gene was monitored in three distinct pomegranate accessions with distinctive skin colour and pattern by semi-quantitative RT-PCR.
Results: The results indicated a strong association between skin colour in mature pomegranate fruits with the *PgMYB* transcripts. The highest expression level of *PgMYB* gene was observed in Poost Siyah Yazd (dark purple skin) throughout the ripening process. Furthermore, comparison of PgMYB amino acid sequences with those of R2R3-MYB family in grapevine, eucalyptus, peach, cacao, populus and Arabidopsis demonstrated that this protein shares high similarity (75-85% amino acid identity) with their conserved MYB domain. Computational structure prediction of PgMYB showed that the three conserved amino acids (Asn, Lys and Lys) are present in the same position of the MYB domain.
Conclusions: It is speculated that *PgMYB* gene influences the fruit colour and could be used to improve the accumula-tion of anthocyanin pigments in the pomegranate fruit.

Keywords: Anthocyanin biosynthesis; MYB transcription factor; Pomegranate

1. Background

Land plants possess successful mechanisms for responding to environmental challenges. In these elaborate systems, transcriptional regulation plays critical roles due to their broad adaptation and coordinately modulates of hundreds of different genes within regulatory networks. Transcription factors are powerful means for regulation of gene expression and consist of at least four distinct domains, DNA binding domain, nuclear localization signal (NLS), transcription activation domain, and oligomerization site, which operate together to control many physiological processes (1). Based on the specific conserved DNA-binding domains, the transcription factor genes can be classified into different families or super-families (2). The MYB transcription factor superfamily is a group of functionally diverse proteins found in both plants and animals. The distinguishing characterization of this family is possession of one to four or more imperfect MYB repeats (R), which can function synergistically

or discretely in DNA binding and protein–protein interactions, respectively (3). The MYB domain consist of tandem repeats of about 50 amino acids located near the protein's amino terminus that is the basis of the classification of this protein family. MYB domain consists of three α-helices, with the second and third helices forming a helix–turn–helix (HTH) structure to intercalate in the major groove of target DNA (1). Activation domain that is resided at C-terminus varies considerably between MYB proteins. This domain plays wide range of regulatory roles in *MYB* gene family (4, 5).

MYB proteins have been categorized into three major groups: R2R3-MYB, with two adjacent repeats; R1R2R3-MYB, with three adjacent repeats; and a heterogeneous group collectively referred to as the MYB-related proteins, which usually but not always contain a single MYB repeat (3-5). In plants, a large number of MYB proteins have essential roles in many significant physiological and biochemical processes, including regulation of primary and secondary metabolisms, control of cell development and cell cycle, and response to various biotic and abiotic stresses (2, 6, 7). In particular, interacting R2R3-MYB and bHLH type TFs, form a complex with WD40 proteins (termed the MBW complex), to control the anthocyanin pathway genes (8, 9). MYB transcription factors that regulate the expression of anthocyanin biosynthetic genes have been identified from many species such as Arabidopsis MYB75 and AtMYB90 (7, 10), petunia AN2 (11), grapevine MYBA1 and MYBA2 (12-14), maize *C1* family (15), apple MYB10/MYB1/ MYBA (16, 17) and legume LAP1 (18).

As powerful antioxidants, anthocyanins exhibit a wide range of protective effects with potential benefits for human health (19). Anthocyanins, as food colourants and beneficial chemo-protective agents are valuable in producing healthier food (20, 21). Anthocyanins, a class of flavonoids with absorbance in the visible range, are also responsible for the red, purple, and blue colours in many fruits, vegetables, cereal grains, and flowers (22, 23). These valuable pigments play important roles in the signaling between plants and microorganisms, plant defense mechanisms, auxin transport, UV protection, recruitment of pollinators and seed dispersers (24). Up to date, the biosynthesis of the anthocyanin pigments have been studied in various plant species (23, 24). The production of anthocyanin is correlated with the activity of enzymes such as phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX, also called anthocyanidin synthase, ANS) and UDP-Glucose: flavonoid-3-*O*-galactosyl transferase (UFGT) in various plants.

Pomegranate (*Punica granatum*) fruits are recognized for their high anthocyanin content accumulation in skin and arils (23, 25). In pomegranate fruits, the degree of pigmentation is crucial factor that determine the appeal of fruits to the customers, influence the antioxidant activity and protect cellular compounds against biotic stress and radiation damage (26, 27). The different pomegranate accessions exhibit variable patterns of skin colour; from yellow, through pink and red to deep purple (28). One possible reason for the observed colour variation in pomegranate skin can be related to differences in the expression of regulatory genes (23). Analysis of pomegranate *MYB* gene showed that the expression level of *PgAn2* is correlated with the skin colour (29). In many plants, different *MYBs* are involved in transcriptional regulation of anthocyanin production. In maize, two classes of regulatory genes (*C1* and *R* gene families) control expression of anthocyanin biosynthetic genes (30, 31). Also, in grapes, *VvMYBA1* and *VvMYBA2* had a regulatory effect on fruit colour (12-14). Therefore, it seems that plant pigmentation patterns are controlled by more than one MYB protein.

In the present work, homologous of *VvMYBA1* and *VvMYBA2* were identified from several plant species, including eucalyptus (*Eucalyptus grandis*) peach (*Prunus persica*), cacao (*Theobroma cacao*), populus (*Populus trichocarpa*) and *Arabidopsis thaliana*. They encode an R2R3 MYB domain protein with high similarity to the grape. An R2R3 MYB regulator was isolated and characterized from pomegranate. The changes in expression of *MYB* was compared during fruit development in three cultivars, namely Shirin Shabad Shiraz (pink), Bozi Isfahan (red) and Poost Siyah Yazd (dark purple).

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Three pomegranate accessions, Poost Siyah Yazd (dark purple skin), Bozi Isfahan (red skin) and Shirin Shabad Shiraz (pink skin) were sampled from the pomegranate tree collection in the Agricultural and Natural Resources Research Center of Isfahan (http://esfahan.areo.ir). Flowers and fruits were collected between May till October. The samples were selected from four different developmental stages:

flower (stage 1), young fruit (stage 2), nearly mature fruit (stage 3) and ripened fruit (stage 4). In Bozi Isfahn, the skin colour in the developing fruit changes gradually from the prominent orange-red (stage 1) to green (stage 2). At fruit maturation and ripening (stage 3 and 4) the colour changed to red. Post Siyah Yazd acquired its black skin very early and remained black until ripening time. In contrast, the colour of Shirin Shabad Shiraz remained green at stages 2 and 3, but turned pink at fruit ripening stage (stage 4). For each sampling stage (1-4), flowers/fruits were collected from three trees and considered as biological replicate. The fruit skin was removed and stored immediately at 80ºC for further analysis.

2.2. Oligonucleotide Design

The degenerate primers were designed according to the conserved regions in R2R3 domain of MYB family from grapevine, eucalyptus, peach, cacao, populus and Arabidopsis that were used for polymerase chain reaction (PCR). To perform semi-quantitative RT-PCR experiments, specific primers were designed from conserved domains of R2R3 MYB protein (Table 1).

2.3. Nucleic Acid Isolation

Total RNA was extracted from Poost Siyah Yazd, Shirin Shabad Shiraz and Bozi Isfahn accessions, from four different developmental stages of flower and fruit skin with the method described for peach trees by (32). Tissues from at least three fruits from three different trees of the same accession and sampling date were combined and extracted. The isolated RNAs were quantified by spectrophotometry (Beckman DU-64 spectrophotometer, USA), the quality was assessed by the absorbance ratios of A260/A280 nm (1.8-2.2) and A260/A230 nm (≥1.8) and by the demonstration of intact ribosomal RNA bands in agarose gel electrophoresis. Each total RNA sample was incubated with 1 U *RNase*-free *DNase I* (Fermentas Co. Germany) for 30 min at 37ºC to remove co-extracted genomic DNA.

Genomic DNA was extracted from fresh young leaves using a CTAB-PVP method as described in (33) and quantified by agarose gel electrophoresis and spectrophotometrically.

2.4. Isolation of PgMYB

A combination of RT-PCR, 3′ and 5′-RACE techniques were used to isolate the full length of *PgMYB*, from Poost Siyah Yazd skin tissue. Initially, a 169 bp fragment was amplified based on the degenerate primers (Table 1). First-strand cDNA was prepared from 1.0 µg of total RNA using MMLV reverse transcriptase and oligo (dT) according to the manufacturer's instructions (cDNA kit; Thermo scientific). PCR conditions were as follows: 95ºC for 4 min followed by 30 cycles of 95ºC (30 s), 58ºC (30 s) and 72ºC (60 s) with a final extension at 72ºC for 7 min and 4ºC for 30 min. DNA fragment is cloned with the InsT/Aclone PCR cloning kit according to the manufacturer's instructions (Thermo Scientific, Germany). Sequence analysis allowed the design of nested primers for 3′ and 5′-RACE reactions (5′/3′ RACE Kit, Roche Applied Science, Germany). To isolate full length sequence of *PgMYB* cDNA.

2.5. Gene Expression Analysis

The expression level of MYB regulator was evaluated using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Samples were normalized with 18S rRNA as a reference gene for constitutive expression. For RT-PCR assays, PCR reactions were performed with 27, 30, 33, and 40 cycles to ensure that amplifications were within the linear range. PCR conditions were as follows: 95ºC (30 s), 58ºC (30 s) and 72ºC (60 s) with a final extension at 72ºC for 7 min. Validation of the correct PCR conditions to appropriately determine level of gene

Table 1. Oligonucleotide primers used for RACE-PCR and RT-PCR experiments

Gene	Name	Sequence (5' to 3')	Annealing temperature (°C)	Amplicon Size (bp)
PgMYB	MYB-1	GCA GGI CTY AAY AGI TGY AG	52	198
	MYB-2	ATT CCA RTA RTT YTT RAT YTC		
	5-MYB	TCT GCA TCT GTT CAG TCC TG	56	123
	3-MYB	GAC TGA TAA CGA GAT TAA GAA C	54	301
PgMYB	MYB-F	GGT TGC AGG ACT GAA CAG ATG C	56	262
	MYB-R	GCT GGA ACT TCT CTG GAT TTC TC		

expression was performed by making serial dilutions of the cDNA samples and consequently the band intensity was determined on 1% (w/v) agarose gels. Densitometric analyses were performed using EZQuant-Gel software. The obtained data were expressed as the mean and standard error (SE) of five PCR replicates.

2.6. In Silico Characterization of PgMYB

To provide comprehensive information for MYB family, the BLAST program was used to search homologues in TF database PlantTFDB version 3.0 (34). A multiple sequence alignment of the deduced amino acid sequence of PgMYB and other MYB proteins associated with regulation of the anthocyanin biosynthetic pathway was constructed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). For pretty printing multiple alignment output, BoxShade program was used (http://www.ch.embnet.org/software/BOX_form.html).

Primary, secondary and tertiary structure analysis of PgMYB and other MYB proteins were performed to identify the structurally similar protein templates on the ExPASy proteomics server (http://www.expasy.org). The tertiary structure of PgMYB was modeled using the tool PHYRE 2.0 (35); PHYRE offered the homology modelling of PgMYB that shared the high similarity with the animal c-MYB DNA-binding domain. The crystal structure of this protein was previously resolved with its DNA consensus motif (AACNG) by heteronuclear multidimensional NMR (36). The Docking studies were used to check the conformation and binding mode of DNA with PgMYB using Hex 6.3 software.

3. Results

3.1. Isolation and Characterization of PgMYB

The full-length size of the *PgMYB* was found to be 930 bp; with 567 bp open reading frame encoding a protein with 189 amino acid residues. Comparison of the deduced PgMYB protein sequence with the R2R3-MYB family in eucalyptus, grapevine, peach, cacao, populus and Arabidopsis revealed that this protein shares high sequence similarity (75-85% amino acid identity) with their conserved MYB DNA-binding domain (Figure 1). Moreover, multiple sequence alignment data showed a high sequence similarity of PgMYB with grape proteins: VvMYBA1 and VvMYBA2 (Figure 1). Additionally, a highly conserved DNA-binding domain was noted at the N- terminus of all the analyzed sequences and for the rest of the sequence of these anthocyanin regulatory proteins the homology was quite high (Figure 1).

Comparison between the genomic DNA and the cDNA sequences (data not shown) indicated that *PgMYB* gene contains a single intron within the protein coding region, similar to *VvMYBA1* isolated from grapevine (37), and other *PgMYB* homologs (Data not shown). *PgMYB* sequence was deposited to GenBank database under accession number KF631413.

3.2. 3D-Structural Modelling of PgMYB

The tertiary structure of DNA-PgMYB complex was predicted based on the experimentally solved DNA-animal c-MYB domain with an E-value of 3.8e-24 within an estimated precision of 99.9% (Figure 2A). The two protein sequences were 35% alike using amino acid sequence alignment. It is intriguing that the animal c-MYB region contains a conserved R2R3-MYB domain that exhibits structural similarity to plant R2R3-MYB DNA binding domains. In fact, this NMR showed that ACG nucleotides in the motif (AACNG) served as the core recognition motif, critical for c-MYB binding. In order to identify target DNA-binding sites for PgMYB, the DNA consensus motif (AACNG) of c-MYB was docked into the predicted binding sites of the PgMYB model (Figure 2B). Based on the previous studies, several specific interactions can be predicted between amino acids and base pairs in the region covered by R2R3 (38-40). In particular, these complex structure is characterized for the specific interactions of the three bases at positions 4, 6, and 8 in the core AACTG; (A4+, G6-, and G8+ are interacting with Asn-183, Lys-182, and Lys-128, respectively) (41). Interestingly, the model of PgMYB revealed that these amino acids were extremely conserved in PgMYB domain and consist of Asn-97, Lys-96, and Lys-42, respectively. In addition, computational structure prediction of the homologues of PgMYB showed that these conserved amino acids are present in the same position of their MYB domain. In fact, the alignment data (Figure 1) demonstrated that the level of conservation in third repeat (R3) is even greater and involves in DNA binding. Several models assumed that both R2 and R3 contain three helices and that each third helix is the recognition helix located in the major groove of DNA. Based on the tertiary structure of DNA-PgMYB complex, three conserved amino acids (Asn, Lys and Lys) involve in DNA binding. Also, these amino acids are conserved in MYB family from eucalyptus, grapevine, peach, cacao, populus and Arabidopsis.

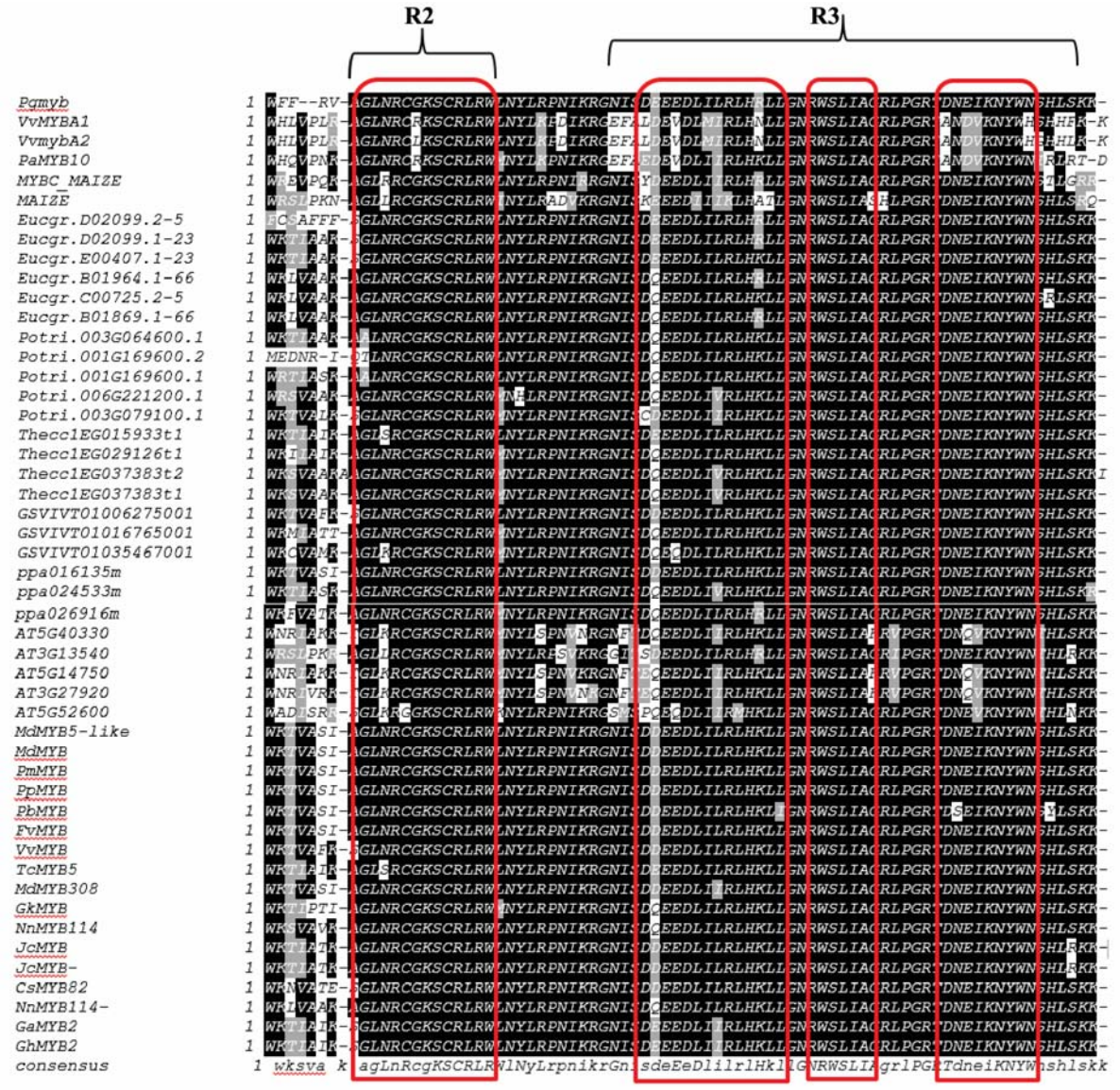

Figure 1. Alignment of deduced amino acid sequences of PgMYB homologous proteins. The R2R3MYB domain are indicated with open boxes. Identical residues are highlighted on a black background, while similar residues and gaps are highlighted on a gray and white background, respectively. The name of the proteins, their source and GenBank accession numbers are as follows: PgMYB isolated from *Punica granatum* (KF631413); VvMYBA1 from *Vitis vinifera* (BAD18977); VvMYBA2 from *Vitis vinifera* (BAD18978); PaMYB from *Prunus persica*; MYBC_MAIZE from *Zea mays* (P10290); Eucgr.D02099.2-5 from Eucalyptus grandis; Eucgr.D02099.1 from *Eucalyptus grandis*; Eucgr.E00407.1 from *Eucalyptus grandis*; Eucgr.B01964.1 from *Eucalyptus grandis*; Eucgr.C00725.2 from *Eucalyptus grandis*; Eucgr.B01869.1 from *Eucalyptus grandis*; Potri.003G064600.1 from *Populus trichocarpa*; Potri.001G169600 from *Populus trichocarpa*; Potri.001G169600.1 from *Populus trichocarpa*; Potri.006G221200.1 from *Populus trichocarpa*; Potri.003G079100.1 from *Populus trichocarpa*; Thecc1EG015933t1 from *Theobroma cacao*; Thecc1EG029126t1 from *Theobroma cacao*; Thecc1EG037383t2 from *Theobroma cacao*; Thecc1EG037383t1 from *Theobroma cacao*; GSVIVT01006275001 from *Vitis vinifera*; GSVIVT01016765001 from *Vitis vinifera*; GSVIVT01035467001 from *Vitis vinifera*; ppa016135m from *Prunus persica*; ppa024533m from *Prunus persica*; ppa026916m from *Prunus persica*; AT5G40330 from *Arabidopsis thaliana*; AT3G13540 from *Arabidopsis thaliana*; AT5G14750 from *Arabidopsis thaliana*; AT3G27920 from *Arabidopsis thaliana*; AT5G52600 from *Arabidopsis thaliana*; MdMYB-like5 from *Malus domestica* (XP_008356551.1); MdMYB from *Malus domestica* (XP_008364573.1); PmMYB from *Prunus mume* (XP_008238440.1); PpMYB from *Prunus persica* (EMJ11205); PbMYB from Pyrus x bretschneideri (XP_009369005); FvMYB from *Fragaria vesca* (XP_004299414); VvMYB from *Vitis vinifera* (XP_010646852); TcMYB5 from *Theobroma cacao* (EOY24284.1); MdMYB308 from *Malus domestica* (XP_008369485); GkMYB from *Gossypioides kirkii* (AAN28279.1); NnMYB114 from *Nelumbo nucifera* (XP_010258843.1); JcMYB from *Jatropha curcas* (AIT52259.1); JcMYB- from *Jatropha curcas* (AIT52258.1); CsMYB from *Citrus sinensis* (XP_006477150); NnMYB114- from *Nelumbo nucifera* (XP_010264377.1); GaMYB from *Gossypium arboretum* (AAU12248) and GhMYB from *Gossypium hirsutum* (AFN53779.1)

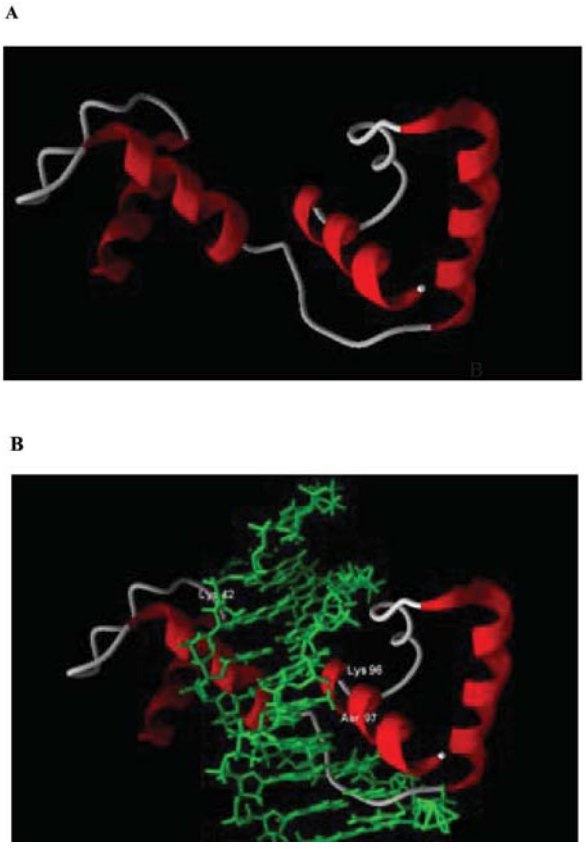

Figure 2. A: The tertiary structure of PgMYB transcription factor, B: The tertiary structure of DNA-PgMYB complex

3.3. Transcriptional Profile of PgMYB Gene

The expression patterns of regulatory gene, *PgMYB* was monitored in three pomegranate accessions and the results are presented in Figure 3. The expression data showed that *PgMYB* transcripts were present at low levels in flowers. In comparison with other accession, Poost Siyah Yazd showed a slight increase of *PgMYB* messenger during the early stages of development (stage 2). However, maximum transcript levels were observed in mature fruits (stage 4). At this stage, the skin colour is very dark purple. In controversy, in Shirin Shaabad Shiraz *PgMYB* transcript was detected at low levels during the fruit development process. In third accession (Bozi Isfahan), *PgMYB* was expressed at low level in young developing fruits (stages 2), but gradually increased as the fruit matured (stage 3). Expression of this gene reached to its peak when the fruit became over-ripened (stage 4). Comparison the steady state level of *PgMYB* mRNA in Poost Siyah Yazd with Bozi Isfahan showed that the expression of this gene was remarkably higher in coloured skin of Poost Siyah Yazd.

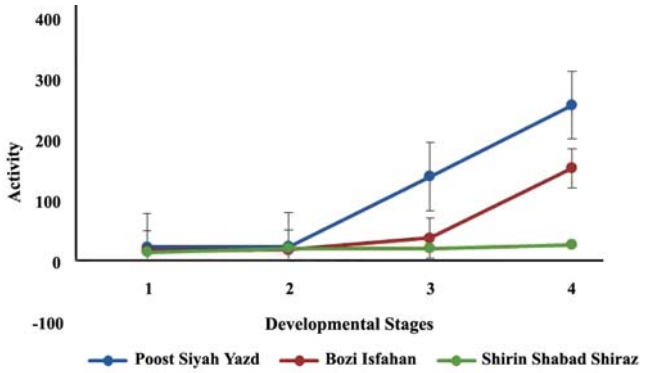

Figure 3. Expression of *PgMYB* gene during fruit development in three different pomegranate accessions, Poost Siyah Yazd, Bozi Isfahn and Shirin Shabad Shiraz. Semi-quantitative RT-PCR analysis was performed and samples were normalized with 18S rRNA as a reference gene for constitutive expression

4. Discussion

MYB/bHLH/WD40 complex is necessary for the appropriate regulation of the anthocyanin biosynthesis various plant species (8). It is known that the difference in the regulatory mechanism mirrors in variation of pigmentation patterns. The present study demonstrated that the highest expression level of *PgMYB* gene was observed in the skin of dark purple fruits (Poost Siyah Yazd). Also, its transcript level increased simultaneously with anthocyanins accumulation in this tissue suggesting that *PgMYB* is involved in anthocyanins biosynthesis in pomegranate fruits. Indeed, the colour of mature pomegranate fruits reflected the activity of regulatory gene *PgMYB* in anthocyanin biosynthesis. Similar results have been obtained in the developmental studies of Arabidopsis (42), maize (15), apple (43), and petunia (11) and grapevine (37). Another point of view, the plant MYB proteins play a crucial role in determining the spatial and temporal patterning of anthocyanin production in most plant species (44). In apple, it was proposed that the MYB component involved in anthocyanins regulation conferred the light responsiveness. In Perilla, MYB factor, MYB-P1, and one of the bHLH factors named MYC-F3G1 are required for anthocyanin synthesis under light condition (45). Moreover, the *MYB* genes *C1* and *Pl* are light induced in maize (15). Further, some members of the MYB family show tissue-specific and developmental pattern of expression. Here, the expression level of regulatory gene *PgMYB* remarkably increased in the skin of pomegranate during fruit

ripening (Poost Siyah Yazd and Bozi Isfahan). These finding are in agreement with Gil's (1995) reports, which showed skin colour continuously enhanced during the maturation of pomegranate fruits (26). In ripening apple fruit the transcription of *MdMYB1* was correlated with anthocyanin production in red skin sectors of fruit (17). Also, in maize, the *C1* gene modulates the expression of structural genes for enzymes involved in anthocyanin production during seed development (15). In other plants, evidence of tissue-specific regulation are reported as well. For instance, *GhMYB7/9* are specifically expressed in flowers and fibers, and their expression in fibers is developmentally regulated (1).

The current study demonstrated that PgMYB protein contains the R2R3 imperfect repeats, responsible for binding to target DNA sequences and is highly conserved among R2R3-MYB proteins. It is well documented that gene-specific regulation is controlled by transcription factors that interact with specific short conserved DNA sequences, known as cis-elemnets (46). Herein, the 3D-structural modelling predicted interactions between PgMYB amino acid residues and corresponding nucleotides in the DNA targets (AACNG). PgMYB transcription regulator like other R2R3MYB gene families in plants is characterized by the MYB domain. This domain consists of two imperfect repeats referred to as R2 and R3, each forming a helix-turn-helix structure of about 53 amino acids (1). In addition, R2 and R3 motifs intercalate in the major groove, so that these recognition helices interact directly with each other to bind to the specific base sequence, AACNG cooperatively (36). Surprisingly, promoter analysis of structural genes involved in anthocyanin pathway showed that CCAAT-box (C**AAC**G**G** motif) is present in promoter region of CHS, F3H and UGFT in Arabidopsis, CHS in grapevine and corn. This conserved motif that is known as stress-responsive element (MYBHv1 binding site) is located upstream of regulatory genes such as *AtMYB12*, *AtMYB113* (42) and *VvbHLH* (47) (data not shown). Based on homology modelling, PgMYB protein bind to this conserved motif, which is present in promoter of structural genes. Therefore, it is expected that PgMYB transcription factor regulate the anthocyanin pathway under developmental stages and/or environmental conditions.

In conclusion, understanding the specific role of MYB to activate pigmentation awaits further experiments. However, it is known that the abundance of anthocyanins in fruits is tightly regulated by three classes of regulatory factors, MYB, bHLH and WD40 proteins. Therefore, PgMYB protein appears to regulate anthocyanins biosynthesis during reproductive stages of pomegranate accessions. The obtained data revealed that PgMYB regulator bind to specific DNA sequences within the promoter regions of anthocyanin biosynthetic genes to enhance their expression. Further, the results showed that the MYB domain is well conserved among plants such as Arabidopsis, grapevine, peach, cacao, populous and pomegranate. The current findings may also facilitate our ability to develop new pomegranate accessions with enhanced anthocyanin content or improved fruit colours by utilizing molecular-genetic approaches.

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