

# Cloning and Expression Analysis of *RrMYB113* Gene Related to Anthocyanin Biosynthesis in *Rosa rugosa*

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## Abstract

Anthocyanin is one of water-soluble natural pigments widely existing in flowers, fruits, stems, leaves and seeds of plants, and it is the major factor conferring pink or red to the petals of *Rosa rugosa*. MYB TFs play an important role in the anthocyanin synthesis in plants. This work aimed to clone the MYB gene related to anthocyanin synthesis in the petals of *Rosa rugosa*, and explore the relationship between them to lay a good foundation for gene engineering improvement of *R. rugosa*. Based on the transcriptional data, a full-length cDNA sequence of MYB Gene, *RrMYB113* (GenBank accession Nos MG720012), was cloned at the first time from the petals of *Rosa rugosa* “Zi zhi” with RT-PCR and RACE methods. The full-length cDNA is 885 bp with an open reading frame of 654 bp, encoding 216 amino acids. The derived *RrMYB113* protein has a molecular weight of 25,297.64 Da, a calculated pI of 9.61, a R<sub>2</sub>R<sub>3</sub>-MYB domain and bHLH binding domain, and it also has the signature motifs ((A/S/G)NDV and KPRPR(T/S)), thus belonging to Sg6 R<sub>2</sub>R<sub>3</sub>-MYB subfamily. In the secondary structure of *RrMYB113* protein, there is 37.04%  $\alpha$ -helix, 39.81% random coil, 14.81% extended peptide chain, and 8.33%  $\beta$ -corner. There is no transmembrane domain and no signal peptide cleavage site, seventeen Ser phosphorylation sites, fifteen Thr phosphorylation sites, four Tyr phosphorylation sites, and no O-glycosylation sites. The expression of *RrMYB113* increased with the color deepening in petals, and it expressed at a higher level in petals than in other tissues of *R. rugosa* “Zi zhi”. These results are meaningful to reveal that *RrMYB113* might be an important regulator in anthocyanin biosynthesis and coloration in the petals of *R. rugosa*.

## Keywords

*Rosa rugosa*, Anthocyanin, R<sub>2</sub>R<sub>3</sub>-MYB, Gene Expression

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## 1. Introduction

*Rosa rugosa* is a deciduous shrub of genus *Rosa* in the *Rosacea* family with highly ornamental value, and it plays an important role in landscaping. The flower color of *R. rugosa* is very single, most of which is red, pink, and white, while other colors are rarely seen, which has seriously limited its application in landscaping. Anthocyanin is one of water-soluble natural pigments widely existing in flowers, fruits, stems, leaves and seeds in natural plants [1] [2] [3] [4], and plays an important role in the color of *R. rugosa*. At present, there are few studies on the molecular regulation mechanism of anthocyanin synthesis in *R. rugosa*. Therefore, cloning the MYB TFs from *R. rugosa* related to anthocyanin synthesis is important for understanding the regulation mechanism of anthocyanin accumulation and changing the colors. Anthocyanin is synthesized through the synthetic pathway of flavonoids in phenylpropane pathway, and it is usually catalysed with a series of synthetase and transport proteins, which most have been cloned from model plants [1] [2] [3] [4], and it has been studied extensively in many plants such as *Petunia hybrid* [5], *Zea mays* [6] and *Malus pumila* [7]. Studies show that R<sub>2</sub>R<sub>3</sub>-MYB, bHLH and WD40 are three important TFs of regulating anthocyanin synthesis in higher plants, and these TFs play a role by forming a transcriptional complex, which is named MYB-bHLH-WD40 (MBW) [1] [4] [8] [9]. As the most widely used transcription factor in anthocyanin synthesis, R<sub>2</sub>R<sub>3</sub>-MYB protein can activate one or more structural genes expression, thereby promoting anthocyanin synthesis [1] [4] [10]-[15]. The *MdMYB1* isolated from *Malus domestica* could induce a large number of anthocyanin to synthesise in cells [16]. The *GhMYB10* isolated from *Gerbera hybrida* is related to the anthocyanin synthesis in petals and leaves, and it can induce the anthocyanin synthesis in pollen sac in the transgenic tobacco [17]. The *VvMYBA1* isolated from *Vitis vinifera* can specifically express in pericarp and could induce anthocyanin biosynthesis [18]. The overexpression of MYB protein encoded by ANT1 in *Lycopersicon esculentum* could activate the expression of CHS, CHI, DFR and other structural genes, thus promoting the anthocyanin synthesis [19].

In this study, we cloned one MYB gene from the petals of *R. rugosa*, and analysed its bioinformatics and expression patterns. These results would provide a theoretical foundation for molecular mechanism of anthocyanin biosynthesis and could be served as the basis for further comprehension of the pigmentation mechanism in *R. rugosa*.

## 2. Materials and Methods

### 2.1. Plant Materials

The plant materials, Chinese representative *Rosa rugosa* “Zi zhi”, “Fen zizhi”, “Bai zizhi”, were from the rose germplasm resources garden at Shandong Agricultural University. *R. rugosa* “Zi zhi” is the most representative traditional rose in China. The stems, leaves, stamens, pistils and petals of these varieties were

collected as samples for expression analysis. All samples were collected directly frozen with liquid nitrogen, and finally stored at  $-80^{\circ}\text{C}$  until used.

## 2.2. Methods

### 2.2.1. Total RNA Extraction and cDNA Synthesis

An EASY spin Plant RNA Kit from Adlai Biotechnology Co., Ltd. was used to extract the total RNA from the tissue in Section 2.1. Agarose gel electrophoresis and spectrophotometer were used to determine the quality and concentration of the RNA. Abm's 5× All-In-One RT MsterMix was used to synthesize the first-strand cDNA.

### 2.2.2. PCR Cloning of Anthocyanin Biosynthesis Related Gene

Based on the related unigene sequences from transcriptome in petals of *R. rugosa*, the specific primers for the anthocyanin biosynthesis related gene were showed in **Table 1**, which were designed with Primer Premier 5.0. PCR amplification was conducted using the synthesized cDNA in Section 2.2.1 as a template and the primers in **Table 1**. The reaction system included 1  $\mu\text{L}$  cDNA, 1  $\mu\text{L}$  F1 primer (10  $\mu\text{mol/L}$ ), 1  $\mu\text{L}$  R1 primer (10  $\mu\text{mol/L}$ ), and 12.5  $\mu\text{L}$  PCR MIX, with ddH<sub>2</sub>O added to a total volume of 25  $\mu\text{L}$ . The reaction conditions were:  $94^{\circ}\text{C}$  for 5 min;  $94^{\circ}\text{C}$  for 30 s,  $53^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 1 min for a total of 35 cycles; and then extension at  $72^{\circ}\text{C}$  for 10 min. Next, 1% agarose gel electrophoresis was used to detect the PCR products. The target PCR fragment was recovered with the Hipure Gel Pure DNA Mini Kit (Magen). The recovered fragment was ligated to the pMD18-T vector and then transformed into *E. coli* DH5a. The positive clones were selected and sent to BGI for sequencing.

### 2.2.3. Bioinformatics Analysis of Gene

BLASTX (NCBI) was used to study the homology of the nucleotide sequence and the deduced amino acid sequence. DNAMAN5.2.2 was used to conduct multiple sequence alignment. The ORF finder (NCBI) was used to search for an open reading frame, and the Conserved Domains database (NCBI) was used to analyze the conserved domains. ExPaSy-SOPMA was used to predict protein secondary structure. The ProtParam Tool was used to analyze protein physical and chemical properties. Furthermore, the ProtScale was used to predict hydrophilic or hydrophobic protein properties. The NetPhos 3.1 Server was used to predict potential protein phosphorylation sites, and the NetOGlyc 4.0 Server was

**Table 1.** Primers used to clone and expression analysis of *RrMYB113* in *R. rugosa*.

Name	Primer sequence (5' to 3')	Purpose
113-F	ATGGAGGTGAGAAAAGGTTCA	Cloning of the Middle Fragment
113-R	TGCGCTTGCTTCTTCTGTAG	
113-3'-F	CCACGAACCTTCACCAAAAGT	3'RACE PCR
B26	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT	
RrMYB113-F	ATGGAGGTGAGAAAAGG	ORF PCR
RrMYB113-R	TTATTGCCTTGCTTCTTCTTG	

used to predict potential protein glycosylation sites. The Neighbor-Joining method from Mega5 was used to create the phylogenetic tree [20] [21].

#### 2.2.4. Real-Time Quantitative PCR Analysis

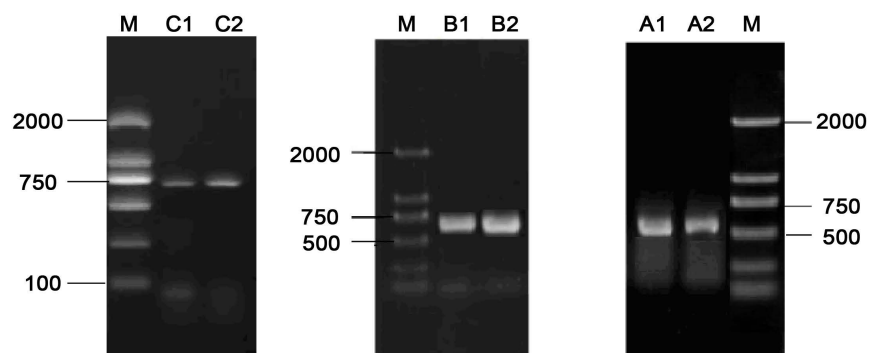
Total RNA extraction and cDNA synthesis were referenced to Section 2.2.1. The expression levels of *RrMYB113* gene involved in anthocyanin biosynthesis were analyzed using quantitative real time PCR. Real time PCR reactions were conducted using two-step PCR System with SYBR Green for detection, using specific primers *RrMYB113-Q-F*(CCACAGTAATAAGACCTCGA) and *RrMYB113-Q-R* (GGTGGTGATGTTGATGATG). The reaction volume was comprised of 20  $\mu$ l containing 10  $\mu$ l SYBR®Premix Ex Taq™, 0.4  $\mu$ l primer (*RrMYB113-Q-F* and *RrMYB113-Q-R*) and 1  $\mu$ l cDNA, with ddH<sub>2</sub>O added to a total volume of 20  $\mu$ L. The reaction conditions were as follows: pre-heating at 94°C for 5 min; 39 cycles at 95°C for 10 s, at 60°C for 30 s. Signals were monitored by the Chromo3 real-time PCR system, finally 30 s at 60°C and 30 s at 95°C for the melting curve. The cycle threshold (Ct) value for each PCR reaction was calculated. After completion of the amplification steps, the melting curve was determined for each analysis. Gene transcripts were quantified using the comparative Ct method, which compares the transcript level of the target gene with that of the reference gene.

### 3. Results and Analysis

#### 3.1. Cloning and Sequence Analysis of *RrMYB113* Gene

One R<sub>2</sub>R<sub>3</sub>-MYB transcription factor, *RrMYB113* (GenBank accession number: MG720012), was cloned from the petals of *Rosa rugosa*, and the blast analysis confirmed that all its homologous genes were R<sub>2</sub>R<sub>3</sub>-MYB TFs. The cloned middle fragment is 625 bp, the cloned 3'-terminal fragment is 498 bp. These two fragments were spliced together with DNASTAR in order to obtain an 885 bp cDNA sequence and the ORF is 651 bp, encoding a polypeptide of 216 amino acids (Figure 1).

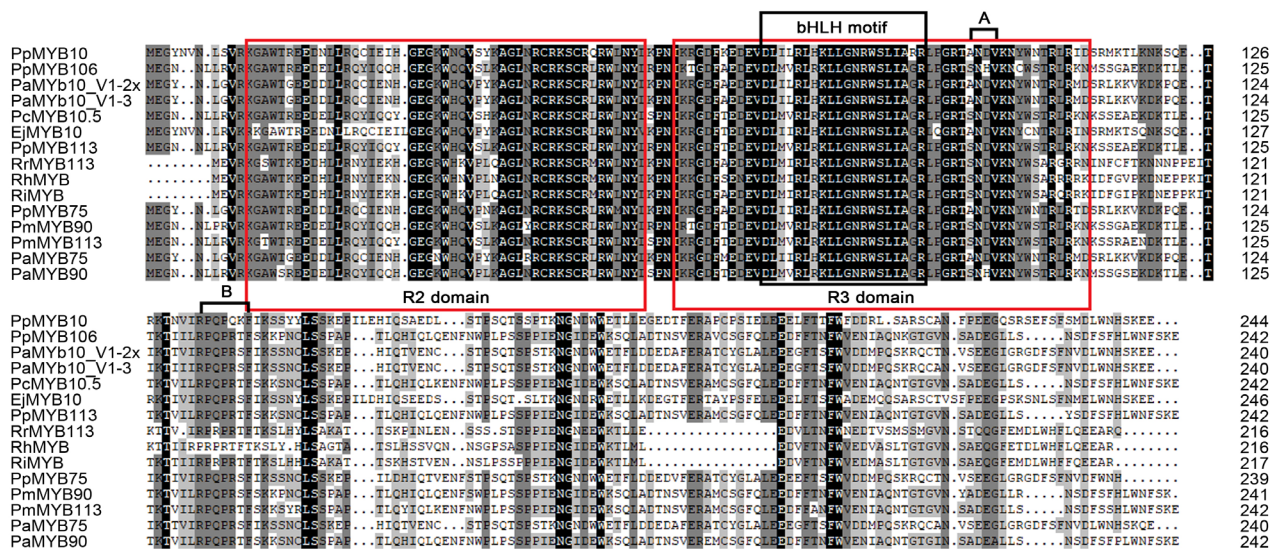
Amino acid sequence alignment between *RrMYB113* and other MYB TFs with



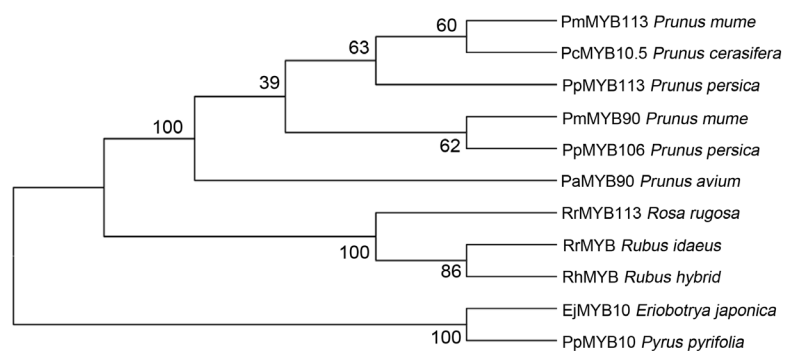
**Figure 1.** PCR amplification of *RrMYB113*. M: Marker; C1, C2: Full-length fragment; B1, B2: Intermediate fragment; A1, A2: 3'-RACE.

higher homology revealed that *RrMYB113* consisted of both R2 and R3 DNA-binding domains. Besides, the alignment showed that the bHLH motif, which interacted with bHLH proteins, appeared in the R3 domain. What's more, *RrMYB113* had the signature motifs ((A/S/G)NDV and KPRPR(T/S)) of Sg6 R<sub>2</sub>R<sub>3</sub>-MYB subfamily (Figure 2).

In order to study the evolutionary relationship between *RrMYB113* and MYB TFs protein in other species, the evolution tree was constructed and analyzed by BLAST with 10 species with homology from high to low. The evolution tree was constructed by MEGA5.0 software, and the system evolution tree was tested by bootstrap, which was repeated 1000 times. The results showed that *RrMYB113* was closely related to the members belonging to *Rosaceae* family, such as *Rubus idaeus*, *Rubus hybrid*, and so on, while it was relatively distant from other MYBs in different families. In addition, eight MYB TFs such as *RrMYB113* and *PaMYB90* were clustered into one branch, and *EjMYB10* and *PpMYB10* gathered into another branch (Figure 3).



**Figure 2.** Multiple alignment of the *RrMYB113* with other MYB TFs. Notes: The red line indicates the conserved R2-domain and R3-domain, the black line indicates the conserved residues interacting with bHLH proteins. Box (A) a conserved motif of [A/S/G]NDV, box (B) a conserved motif of KPRPR(T/S).



**Figure 3.** The phylogenetic tree derived from the alignment of amino acid sequences of *RrMYB113* and other MYB TFs.

### 3.2. Bioinformatics Analysis of *RrMYB113* Gene

The *RrMYB113* protein encoded 216 amino acids, 35 basic amino acids (Arg + Lys), 27 acid amino acids (Asp + Glu), and 154 neutral amino acids, and the prediction molecular formula was  $C_{1109}H_{1747}N_{333}O_{329}S_9$ . The derived protein had a molecular weight of 25,297.64 Da, a calculated pI of 9.61. It belonged to the unstable protein with an unstable index at 59.15, and it was also a hydrophilic protein with the total average hydrophobic index at  $-0.906$ . The secondary structure prediction result demonstrated that there were 37.04%  $\alpha$ -helix, 39.81% random coil, 14.81% extended peptide chain, and 8.33%  $\beta$ -corner. The phosphorylation site prediction results demonstrated that there were 17 Ser phosphorylation sites, 15 Thr phosphorylation sites, 4 Tyr phosphorylation sites, and no O-glycosylation sites.

### 3.3. Expression Patterns of *RrMYB113* in Different Tissues and Different Varieties

The expression analysis of *RrMYB113* in different tissues showed that *RrMYB113* expressed differentially among stems, leaves, stamens, sepals, pistils and petals. *RrMYB113* was more abundant in petals than stems, leaves, stamens, sepals and pistils. The highest expression level of *RrMYB113* was observed in petals, while it expressed slightly in pistil, stamen and leaves, and almost didn't express in sepals and stems. In addition, the results showed that the expression of *RrMYB113* increased with the color deepening among the three cultivars, highest in *R. rugosa* "Zi zhi", followed by *R. rugosa* "Fen zizhi", and the lowest in *R. rugosa* "Bai zizhi" (Figure 4).

## 4. Discussion

In this study, a MYB gene named *RrMYB113* has been isolated from *R. rugosa*.

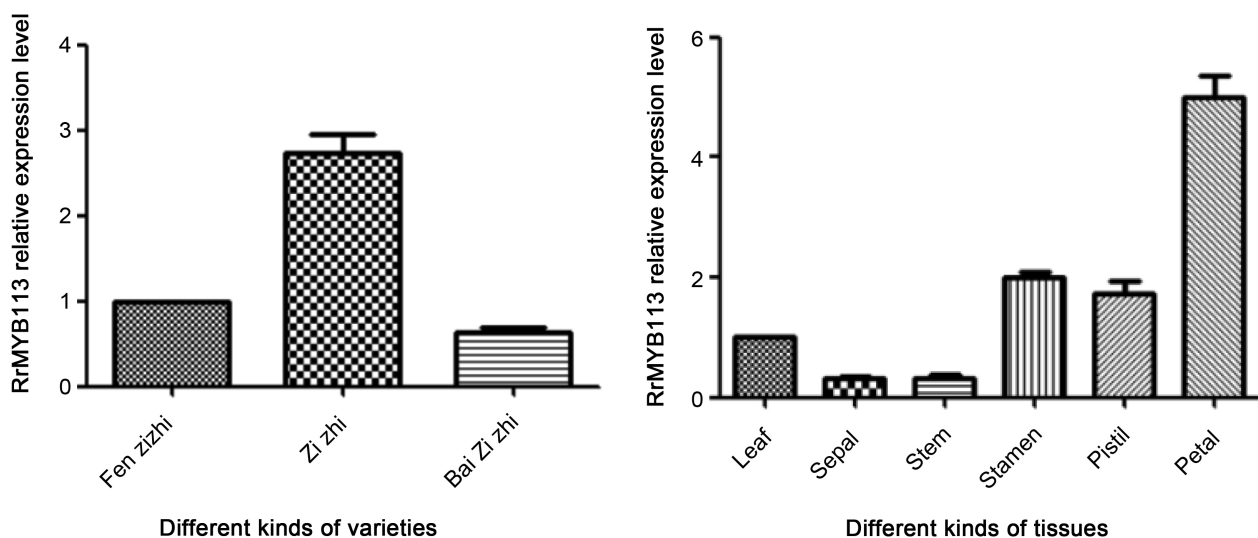


Figure 4. Relative expression levels of *RrMYB113*.

The amino acid sequence alignment showed that *RrMYB113* contained R2 and R3 DNA-binding domains, and it also had a bHLH interaction motif in the R3 domain, which provided corresponding binding sites for the formation of the three element complex (MYB-bHLH-WD40) [1] [6]. Furthermore, it had the signature motifs ((A/S/G)NDV and KPRPR(T/S)), and belonged to Sg6 R<sub>2</sub>R<sub>3</sub>-MYB subfamily. Previous studies show that the R<sub>2</sub>R<sub>3</sub>-MYB proteins of the Sg6 subfamily are mainly involved in the regulation of the synthesis and accumulation of anthocyanins in plants [4]. Many R<sub>2</sub>R<sub>3</sub>-MYB TFs are known to control anthocyanin biosynthesis by regulating structural genes in the anthocyanin pathway [14] [22] [23]. At present, R<sub>2</sub>R<sub>3</sub>-MYB TFs of Sg6 subfamily have been cloned from *Rosa chinensis*, *Lycopersicon esculentum*, *Dioscorea esculenta*, *Malus domestica*, *Citrus sinensis*, and so on [12] [14] [24] [25]. In several other plant species, the expression of many R<sub>2</sub>R<sub>3</sub>-MYB TFs in the anthocyanin pathway is strongly correlated with anthocyanin accumulation. For example, *MdMYB10* express highly in red-fleshed apple, but is virtually undetectable in the white-fleshed apple [26]. Evolutionary analysis showed that *RrMYB113* was highly homologous to the MYB TFs of the Sg6 subfamily in other species. Therefore, it's conjectured that the *RrMYB113* gene was related to anthocyanin synthesis.

Through the bioinformatics analysis, we found that the alpha helix and random coil accounted for a considerable proportion in the secondary structure of *RrMYB113* protein, while the extended strand and beta turn occupied small percentage. A previous study has reported that the alpha helix plays an important role in R motif of the MYB domain, and each R motif is generally composed of three alpha helices, and the second and third R motif form a HTH structure and then combine with the first R motif, further forming a HTH domain with a hydrophobic core. What's more, the third alpha helices in R motif has a role of identifying DNA, so that the MYB protein has high specificity. Therefore, it was predicted that the *RrMYB113* gene belonged to the R<sub>2</sub>R<sub>3</sub>-MYB [9]. Besides, the random coil is beneficial to the combination of cells with water, and *RrMYB113* belongs to the hydrophilic protein, so we presumed that *RrMYB113* gene may play a protective role in osmotic stress of plants [27].

The results of Real-time quantitative PCR showed that the expression of *RrMYB113* gene exhibited a decreasing trend in the petals of *R. rugosa* "Zi zhi", *R. rugosa* "Fen zizhi" and *R. rugosa* "Bai zizhi", and in the expression of different tissues in *R. rugosa* "Zi zhi", the *RrMYB113* gene highly expressed in petals, while in a very low level in other tissues. Previous studies indicate that there are positive and negative mechanisms of MYB protein on anthocyanin regulation in plants [9]. For example, in apples, the *MdMYB1* is positively related to anthocyanin synthesis, and it is regulated by light. And overexpression of *MdMYB10* which cloned from leaf and pulp could increase the accumulation of anthocyanin in seedlings, while overexpression of *MdMYB16*, *MdMYB17* and *MdMYB111* in tobacco could inhibit the activity of DFR promoter, and then influence the

anthocyanin synthesis [16] [28]. And the *FaMYB10* gene isolated from *Fragaria × ananassa* is similar to *MdMYB10*, which could promote the anthocyanins synthesis, while the *FaMYB1* gene exhibit oppositely [15]. In the present study, the expression level of *RrMYB113* increases with the color deepening, and it's highest expressed in the petals of *R. rugosa* “Zi zhi” in different tissues. Therefore, the author believed that the *RrMYB113* gene positively regulate the anthocyanin synthesis in *R. rugosa*.

## 5. Conclusion

In conclusion, one R2R3-MYB TF, *RrMYB113*, was isolated from *R. rugosa* and was found to be involved in regulating anthocyanin biosynthetic pathway. The results of this study provided important information on the anthocyanin synthesis of *R. rugosa*. In future work, we will test whether the overexpression of *RrMYB113* leads to anthocyanin accumulation in *Arabidopsis thaliana* and *Nicotiana tabacum*.

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