Effect of pH on the expression of *RsMYB1* that regulates anthocyanin production in *Petunia* plants

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Abstract We established an *in vitro* system to investigate transcription levels of the *RsMYB1* gene expressed in T2 20-day-old transgenic *Petunia* plants (three independent lines: *PhRs1*, *PhRs2*, and *PhRs3*), and the association between those transcription levels and anthocyanin production at various pH values (3.0 to 8.0) for a period of 10 days. All the lines treated with pH 5.0–7.0 exhibited increased anthocyanin content and delays in growth compared to the wild-type (WT) seedlings. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis confirmed that the enhancement of anthocyanin production in the transgenic lines was due to the upregulation of *RsMYB1* transcription at various pH values. The results suggest that pH value can control expression of *RsMYB1* which is associated with anthocyanin production.

Keywords *Petunia*, Anthocyanin, pH, qRT-PCR, *RsMYB1*

Introduction

Anthocyanins are well-known water-soluble pigments that impart colour to plants (Byamukama et al. 2011; Li et al. 2013), including their fruit, flowers, leaves, stems, and occasionally roots (Davies 2004; Andersen and Jordheim 2006). Anthocyanins protect plant tissues and senescing autumn leaves against damaging photo-oxidation, promote pollination, and facilitate seed distribution. The accumulation of anthocyanin pigments depends on factors such as temperature, light, osmotic stress, co-pigments, and pH (Rodriguez-Saona et al. 1999). Antho-

cyanins usually accumulate in the vacuoles of epidermal cells, and their colour depends on the pH of the vacuole in which they localize.

Enhancement of anthocyanin production by the different stress conditions, such as high-intensity light, low temperatures, nutrient deficiency, or pathogen attack, and pH stress, had been observed in many plant species (Dixon and Paiva 1995; Chalker-Scott 1999, Zhang et al. 2014). However, role of pH in anthocyanin accumulation have not been well demonstrated in *petunia*. Recently, Ai et al (2016) claimed that overexpression of *RsMYB1* increased anthocyanin accumulation in vegetative and floral tissue of transgenic *petunia*. Hence, it was of interest to investigate the interaction between pH values and *RsMYB1* expression controlling anthocyanin production of the transgenic *petunia*.

In this study, we used the transgenic plant ('Mirage Rose') expressing *RsMYB1* to investigate the morphological variation and anthocyanin accumulation under different pH conditions.

Materials and Methods

Plant materials

Seeds of three independent T₂ transgenic *Petunia* lines (*PhRs1*, *PhRs2*, and *PhRs3*) and its wild-type (WT) used in this experiment was provided by Dr. Trinh Ngoc Ai (the Department of Horticultural Science, Kyungpook National University, Daegu, South Korea). The seeds were sterilized in 0.05% sodium hypochlorite solution (Yuhan Co, Ltd., Seoul, South Korea) containing 0.01% Tween 20 (Duchefa Biochemie, Haarlem, The Netherlands) for 10 min and rinsed several times with sterile distilled water. The sterile seeds were sown in Murashige and Skoog (MS) medium containing 3% sucrose and 0.3% Gelrite™ (pH 5.8). The cultures were incubated at 25 ± 2°C and subjected to a 16-h photoperiod at an intensity of 50 μmol·m⁻²·s⁻¹ for 20 days.

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Table 1 List of primers used in the quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of anthocyanin biosynthesis

Gene	Primer sequences
<i>TUB</i>	FP: 5'-TGG AA CTC AAC CTC CAT CCA-3' RP: 5'-TTT CGT CCA TTC CTT CAC CTG-3'
<i>CHS</i>	FP: 5'-GAA CAG CCA CAC CTA CAA AC-3' RP: 5'-AAC CCT GCT GGT ACA TCA TG-3'
<i>CHI</i>	FP: 5'-TTC CAC CGT CCG TCA AAC CT-3' RP: 5'-CAT GCC TCC ACT GCA ACC AC-3'
<i>FLS</i>	FP: 5'-CCG ATT TGG CTC TTG GTG TT-3' RP: 5'-TTT GGG ACA AGA ATG GTG ATA TAT GA-3
<i>DFR</i>	FP: 5'-AGC AGG AAC TGT GAA TGT GG-3' RP: 5'-GTT GGG ATA ATG GTG ATG AAA T-3'
<i>ANS</i>	FP: 5'-TGG GAG GAT TAT TTC TTC CA-3' RP: 5'-GTT GTA CTT GCC GTT GCT TA-3'
<i>DPL</i>	FP: 5'-TTT GTC CGG AGG AAG TGG AC-3' RP: 5'-CGG AAG TCT CCC GGC AAT AA-3'
<i>PHZ</i>	FP: 5'-GAT GGT CAC TTA TCG CGG GT-3' RP: 5'-TCC TCT GCA GGT GTG TGT TC-3'
<i>JAF13</i>	FP: 5'-ACG GAT GAT AAT ATG AGT AAC GGT GTG C-3' RP: 5'-CTT GAT GGT CTA GTG GGG CAG GC-3'
<i>RsMYB1</i>	FP: 5'-ATG GAG GGT TCG TCC AAA GG-3' RP: 5'-GAA ACA CTA ATC AAA TTA CAC AGT CTC TCC-3'

Effect of pH on anthocyanin accumulation and gene expression

To determine the effect of the pH on pigment content, 20-day-old transgenic seedlings were cultured in liquid MS media containing the different pH values (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0). The cultures were incubated at $25 \pm 2^\circ\text{C}$ and subjected to a 16-h photoperiod at an intensity of $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. After 10 days of culture, the seedlings were transferred to new media for next 10 days. After which, RNA samples were collected to investigate the regulation of anthocyanin gene biosynthesis. The effect of pH on anthocyanin content was also monitored in the transgenic seedlings cultured in the media containing different pH values. We performed anthocyanin content analysis and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis according to the protocols described by Ai et al. (2016). The qRT-PCR primers are listed in Table 1.

Effect of pH on the development of transgenic lines

After treatment of 20 days, 10 plants were randomly selected to determine the effect of pH on transgenic lines and compared with the WT. The breadth of the leaves and the root length were measured to the nearest mm with a rule (Absolute Digimatic, Japan). The numbers of shoots and leaves were also recorded.

On Day 20, the average areas and the length/breadth measurements of 10 replicates of the fully developed leaves were measured to represent the size and shape of the leaves, respectively. The breadth was measured from the centre of the midrib region. The experiments were repeated three times, and standard errors were determined for the data obtained from each experiment.

Data analysis

All data were subjected to one-way analysis of variance (ANOVA) and the Duncan's SSR test (shortest significant ranges) to compare differences between the experimental sites at $P < 0.05$ (Microsoft Excel 2003 and SAS 9.4 software).

Results and Discussion

Effect of pH on the development of transgenic *Petunia* seedlings

We determined the effect of media pH (six different pH values) on the development of transgenic lines of *Petunia* seedlings. After 20 days of culture, there are significant differences in growth between the WT and the transgenic plants. The num-

Table 2 Growth parameters of seedlings after 20 days of treatment at various pH values in wild-type (WT) and transgenic plants. Numbers of shoots and leaves, leaf sizes, and root lengths were determined. The data are the means of 10 replications

Morphological line characteristics	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
Number of shoots						
WT	1.0 ± 0.0e	1.2 ± 0.2e	1.0 ± 0.0e	1.2 ± 0.2e	1.8 ± 0.4e	2.8 ± 0.8de
<i>PhRs1</i>	2.8 ± 0.9de	7.2 ± 0.6bcd	6.8 ± 0.9bcd	5.2 ± 0.4cde	3.6 ± 0.5de	3.4 ± 0.8de
<i>PhRs2</i>	2.0 ± 0.6e	3.4 ± 0.9de	3.4 ± 0.9de	3.8 ± 0.5de	3.8 ± 0.8de	5.0 ± 1.1de
<i>PhRs3</i>	3.6 ± 1.0de	9.6 ± 4.3ab	7.4 ± 1.3bcd	9.4 ± 1.9abc	12.2 ± 2.6a	13.6 ± 2.5a
Number of leaves						
WT	8.0 ± 0.8f	14.4 ± 1.7dfce	13.4 ± 2.8dfce	14.0 ± 0.7dfce	13.4 ± 1.2dfce	17.0 ± 1.8dce
<i>PhRs1</i>	10.4 ± 1.2fe	11.4 ± 1.8dfe	17.8 ± 3.67dc	17.0 ± 1.1dce	16.4 ± 4.2ce	19.2 ± 3.3bc
<i>PhRs2</i>	11.0 ± 0.5dfe	13 ± 1.1dfce	13.8 ± 2.5dfce	15.8 ± 0.7dce	15.4 ± 2.4dce	13.2 ± 1.1dfce
<i>PhRs3</i>	11.8 ± 1.3dfe	14.6 ± 1.3dfce	10.8 ± 0.6dfe	15.2 ± 1.2dce	24.2 ± 3.1ab	25.5 ± 1.3a
Leaf size (mm)						
WT	174.9 ± 51.3i	373.4 ± 39.6hi	631.2 ± 50.4de	759.5 ± 81.5ab	689.3 ± 47.6cd	697.9 ± 77.2c
<i>PhRs1</i>	332.5 ± 68.1hi	465.5 ± 78.5fg	450.8 ± 135.9fg	543.5 ± 78.7ef	575.2 ± 41.4ef	673.1 ± 96.5de
<i>PhRs2</i>	198.4 ± 21.1hi	484.4 ± 67.1fg	433.4 ± 108.2gh	571.7 ± 62.3ef	363.38 ± 57.1hi	442.1 ± 53.6gh
<i>PhRs3</i>	264.5 ± 43.1hi	627.8 ± 51.3de	380.6 ± 68.0hi	804.3 ± 132.3a	796.33 ± 86.1a	327.5 ± 53.6hi
Root length (mm)						
WT	8.94 ± 2.1f	93.9 ± 13.3cd	138.4 ± 6.18a	99.9 ± 22.1bcd	126.8 ± 17.8ab	97.5 ± 12.5cd
<i>PhRs1</i>	0.0 ± 0.0f	82.5 ± 15.2de	76.5 ± 12.62de	123.9 ± 7.6abc	107.2 ± 10.1cd	101.0 ± 7.5cd
<i>PhRs2</i>	6.68 ± 6.8f	94.5 ± 7.4cd	102.2 ± 10.8cd	105.2 ± 8.2cd	87.4 ± 10.1dc	83.1 ± 15.9de
<i>PhRs3</i>	13.6 ± 9.1f	89.2 ± 5.2dc	70.1 ± 1.31de	86.6 ± 11.4dc	79.7 ± 8.1de	48.4 ± 16.1e

bers of shoots and leaves, the sizes of the leaves, and the lengths of the roots are given in Table 2, whereas the numbers of shoots and leaves were significantly higher in the transgenic plants compared to that in the WT plants. These results indicate that the optimum pH value for the growth of transgenic *Petunia* seedlings is 6.0–8.0, and an acidic environment inhibits the development of seedlings.

The leaves of the transgenic seedlings were smaller than those of the WT seedlings, but there were more leaves on the transgenic seedlings. Similar patterns have been detected in *Arabidopsis thaliana* and in maize (*Zea mays*) (Wang et al. 2011). Anugoolprasert et al. (2012) reported that the optimum pH for the development of crab apples was 6.0 owing to anthocyanin accumulation. However, in the present experiment pH values over 7.0 were associated with the strongest development. We assume this difference was due to the use of different cultivars of crab apple and *Petunia* in the present study.

Accumulation of anthocyanin responds to pH in transgenic *Petunia* seedlings

We used a spectrometer to determine anthocyanin accumulation in transgenic *Petunia* seedlings at various environmental pH values. We used 0.5 g of leaves after 20 days of cultivation. Three transgenic lines (*PhRs1*, *PhRs2*, and *PhRs3*) exhibited different levels of anthocyanin expression. As shown in Figure 1, anthocyanin accumulation was visually observable in the three transgenic lines, but not in the WT seedlings. The pigment was clearly observed when seedlings were cultured

in environments with higher pH values (pH 6.0–8.0). The leaves collected from the WT seedlings were green, whereas those from the transgenic seedlings were red (Fig. 1). The anthocyanin levels were significantly higher in the transgenic plants than in the WT plants (Fig. 2). Of the three transgenic lines, anthocyanin content was highest in *PhRs3* over all pH values, however, the anthocyanin levels in the WT seedlings remained unchanged at all pH values.

Bakowska et al. (2003) reported that a pH of 3.5 was suitable for anthocyanin co-pigmentation in Japanese morning glory (*Ipomea nil*), and a pH of 4 can affect anthocyanin accumulation in *Petunia* plants (Tanaka et al. 2000; Quattrocchio et al. 2006; Koes et al. 2005). Our results indicated that enhancement of anthocyanin in three transgenic lines seemed to be associated with pH, but such association was not observed for WT plants. Higher anthocyanin accumulation was clearly observed when the seedlings from all the transgenic lines were cultured in media with higher pH values.

Effect of pH on the expression levels of anthocyanin biosynthesis genes

According to results of qRT-PCR, expression of anthocyanin biosynthesis genes in the three transgenic lines and the WT line had the same expression trends (Fig. 3), and that higher anthocyanin accumulation was associated with higher levels of gene expression. The *PhRs3* line exhibited the highest levels of expression. We found low levels of expression in the WT line. Transcription levels of the anthocyanin biosynthetic structural genes *CHS* and *CHI*, which are involved in the first

Fig. 1 Anthocyanin accumulation after 20 days of treatment at various medium pH values in wild-type (WT) and transgenic plants

Fig. 2 Accumulation of anthocyanin at various pH values in wild-type (WT) and transgenic seedlings after 20 days of treatment. The data are the means of three replications, and the bars indicate standard error

reaction of the anthocyanin biosynthetic pathway, were significantly increased in the *PhRs2* and *PhRs3* lines at pH 6.0–8.0, compared to those in the WT seedlings. The expression levels of *DFR* and *ANS* (which control the last step in the biosynthetic anthocyanin pathway and encode anthocyanin or flavonol biosynthesis) were significantly higher in *PhRs2* and *PhRs3* than in *PhRs1* or the WT plants. Expression levels of

anthocyanin biosynthetic structural genes *ANS* and *JAF13* (responsible for the last reaction in the anthocyanin biosynthetic pathway) were significantly higher in *PhRs2* and *PhRs3* at higher pH values, compared to those in the WT seedlings. The expression levels of *DFR* and *FLS*, which control the biosynthetic direction of the anthocyanin and flavonol pathways, encoding anthocyanin or flavonol biosynthesis, were significantly higher in *PhRs2* and *PhRs3* than in *PhRs1* or the WT plants. Zhang et al. (2014) indicated that, under high pH condition, the high expression levels of flavonol biosynthetic genes are associated with flavonol accumulation in crab apple cultivars, grapevine leaves (Gutha et al. 2010), and Kiwifruit (*Actinidia*) (Fraser et al. 2013).

The present study showed that in transgenic plants expressing the *RsMYB1* transcription factor, the content of anthocyanin pigments increased significantly at low pH values in vegetative plants owing to the expression of anthocyanin biosynthesis genes, and high pH values increased the growth parameters. However, enhancement of anthocyanin significantly increased the number of shoots and leaves. In contrast, the leaf size decreased. Furthermore, anthocyanin accumulation dramatically (and visibly) increased in the *RsMYB1* transgenic plants.

Fig. 3 Effect of pH on the expression of anthocyanin-related genes in wild-type (WT) and transgenic seedlings. Gene expression levels were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR), as described in the text. The data are the means of three replications, and the bars indicate standard error

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