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Sucrose Enhances Anthocyanin Accumulation in Torenia by Promoting Expression of Anthocyanin Biosynthesis Genes

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Abstract: We examined the effects of different sucrose concentrations (3%, 5%, and 7%) on anthocyanin accumulation and plant growth in wild type (WT) and transgenic (T_2) torenia cultivar "Kauai Rose" overexpressing the anthocyanin regulatory transcription factors B-Peru + mPAP1 or RsMYB1. Sucrose increased anthocyanin production in both WT and transgenic plants, with higher anthocyanin production in transgenic plants compared to WT plants. Higher sucrose concentrations increased production of anthocyanin in transgenic and WT plants, with increased anthocyanin production associated with increased expression of anthocyanin biosynthesis genes. Higher sucrose concentrations reduced growth of WT and transgenic plants. Our results indicate that sucrose enhances anthocyanin production in torenia by regulating anthocyanin biosynthesis genes.

Keywords: anthocyanin; gene expression; plant growth; sucrose; transgenic plant

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1. Introduction

Anthocyanins are responsible for pigmentation (ranging in colors from red to purple) of vegetative (leaf, stem, and roots) and reproductive (flower and fruit) organs of many plant species. Color, particularly in flowers and fruits, contributes to desirability to customers. Presence of anthocyanins in fruits and vegetables may enhance pharmaceutical properties for human health and protect plants from oxidative stresses [1,2]. Anthocyanin synthesis is regulated by environmental factors and hormones [2]. Sugars have been shown to stimulate the growth and storage of sink tissues and have endocrine activity in the regulation of photosynthesis, nutrient mobilization, and allocation [3,4]. In addition, increased anthocyanin production in response to sugar has been demonstrated in flowers, fruits, and vegetative tissues [5–16]. Sucrose stimulates anthocyanin production via upregulation of anthocyanin-related genes. Exogenous sucrose and accumulation of endogenous sugar have been shown to increase expression of early and late anthocyanin biosynthesis genes (EBGs and LBGs), including chalcone synthase (CHS), dihydroflavonol reductase (DFR), and anthocyanidin synthase (ANS), in grape, petunia, and radish [5,6,15,16]. Moreover, sucrose has been shown to increase levels of MYB75/PAP1, a transcription factor (TF) required for expression of *DFR* in petunia [17] and expression of the transgene, *Petunia hybrida*; *PhCHS*, in transgenic *Arabidopsis* leaves [14]. However, Ai et al. [18] reported that sucrose stimulates anthocyanin production in transgenic petunia overexpressing R2R3MYB TF isolated from *Raphanus sativus* (RsMYB1) or overexpressing both bHLH TF isolated from corn (B-Peru} and R2R3MYB TF isolated from *Arabidopsis* (mPAP1) but not in wild type (WT) plants. Hara et al. [7] also reported that sucrose-induced anthocyanin accumulation was dependent on cultivars in radish, whereas sucrose remarkedly upregulated anthocyanin-related genes in red radish but not in white radish. Therefore, the regulatory mechanisms

underlying sugar-induced anthocyanin biosynthesis remain unclear. Previous studies have demonstrated that 7% sucrose can induce anthocyanin in torenia shoots by degrading chlorophyll contents [10,19], whereas anthocyanin induction did not occur when the shoots were retransferred to 1.5% sucrose. However, expression levels of anthocyanin-related genes in response to sucrose were not evaluated in this study. Recently, Junping et al. [20] reported individual overexpression of the RsMYB1 or combinatorial overexpression of the TFs (B-Peru+mPAP1) enhance anthocyanin accumulation via upregulation of anthocyanin biosynthesis genes, despite no significant visible in phenotype.

In our previous work, we produced transgenic Petunia plants expressing the transcription factors R2R3MYB (mPAP1) and bHLH (B-Peru) in combination

Sucrose-induced anthocyanin accumulation in shoots is associated with nitrogen deficiency, with nitrogen deficiency shown to contribute to accumulation of anthocyanins in *Arabidopsis* (WT) and pap1-D mutants via upregulation of PAP1 and PAP2 [21]. Higher sucrose concentrations cause increased nitrogen deficiency, thereby resulting in increased anthocyanin accumulation and chlorophyll degradation in addition to reduced plant growth [9,11,13,19]. Sucrose-induced anthocyanin production is reported to be associated with increased antioxidant activity, including increased levels of superoxide dismutase, peroxidase, and catalase [8,22].

In the present study, we investigated the role of sucrose in anthocyanin accumulation in torenia (WT) plants and transgenic torenia overexpressing RsMYB1 or B-Peru and mPAP1 by measuring anthocyanin-related gene expression. In addition, we evaluated the effect of sucrose-induced anthocyanins on plant growth in vitro and the antioxidant activity of anthocyanins.

2. Materials and Methods

T_2 seeds of torenia transgenic lines '*Torenia fournieri* cv. Kauai Rose' overexpressing RsMYB1 (two independent Lines: Rs1 and Rs2) and B-Peru + mPAP1 (two independent Lines: BM1 and BM2) were provided by Junping et al. [20]. The MYB transcription factor RsMYB1 or mPAP1 was isolated from *Raphanus sativus* or *Arabidopsis*, and the bHLH transcription factor B-Peru was isolated from corn. The transcription factor genes were placed under the control of the 35S cauliflower mosaic virus (35S CaMV) promoter.

2.1. Effect of Sucrose on Anthocyanin Accumulation and Plant Growth

T_2 and WT seeds were germinated on hormone-free MS basal medium. Healthy and uniform germinated seedlings at a height of ~10 mm were then transferred to the same media containing different concentrations of sucrose (3%, 5%, or 7%). As the concentration of sucrose (3%) was widely used as recommended concentration for in vitro culture, it was used as a control in this work. In addition, the concentration of sucrose (7%) was also reported to induce anthocyanins in previous studies [10,19]. The sucrose (5%) may be considered as intermediate concentration. Therefore, the different concentrations of sucrose (3%, 5%, or 7%) were used in this study. Each treatment conducted 30 seedlings, and there were 3 replicates per treatment. Plants were cultured at room temperature with a photoperiod of 16 h. After 3 weeks of culture, plant growth parameters (plant height, root length, and fresh weight) were determined from 30 randomly selected seedlings from each treatment. A group of 10 seedlings is regarded as one replication. Plant height and root length were measured to the nearest millimeter using an automatic ruler (Absolute Digimatic, Kanagawa, Japan).

2.2. Analysis of Total Anthocyanin Content

Anthocyanin content analysis was performed following the procedure described by Ai et al. [18]. Briefly, leaf material (500 mg) from transgenic and WT seedlings was crushed in a mortar with liquid nitrogen to obtain fine powder. Fine powder was then transferred to an extraction solution (5 mL of 1% (*w*/*v*) hydrochloric acid in a methanol solution), and the mixture was incubated at 4 °C for 24 h and then centrifuged at 13,000 rpm

for 20 min at 4 °C. The supernatant was then transferred to a fresh tube and the total anthocyanin content was determined by measuring the OD at A530 (max for anthocyanin) and A657 (peak of absorption for chlorophyll) using a spectrophotometer (Shimadzu, Kyoto, Japan). Quantification of anthocyanins was performed using the formula: Qanthocyanins = (A530 − 0.25 × A657) × M − 1, where Qanthocyanins is the number of anthocyanins, and M is the weight (g) of the plant material used for extraction, as described by Chu et al. [23]. Three different biological samples (three leaves) were used for this analysis. One sample (one leaf) is regarded as one replication.

2.3. RNA Extraction and Anthocyanin Biosynthesis Gene Expression Analysis

Total RNA was isolated from the leaves of transgenic and WT seedlings using RNAqueous kits (Ambion Inc., Austin, TX, USA). Reverse transcription was performed using 1 µg of total RNA and an oligo dT20 primer, according to the manufacturer's instructions (ReverTra Ace-aı, Toyobo, Japan). Sequences for the anthocyanin biosynthesis genes [*TfCHS* (AB548586.1), *TfF3H* (AB548588.1), *TfF3′5′H* (AB548590.1), *TfDFR* (AB548587.1), *TfANS* (AB548583.1), and *Tf3GT* (AB548591.1)] were obtained from the study of Sasaki et al. [24] and their transcript levels were detected using a StepOnePlus Real Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA), and the actin3 (*ACT3*; AB330989.1) gene was used as an internal standard control (Sasaki et al. 2016) [24]. Primers used for detection of the genes are described in Table 1. PCR condition used for this analysis is 40 cycles of 95 °C for 30 s, 58 °C for 30 s, 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Three different biological samples (leaves) were used for this analysis. One sample is regarded as one replication.

Table 1. List of primer accession number and sequences used in quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Genes	Accession No.	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
TfCHS	AB548586.1	TGAGCGAGTACGGGAACATG	TTCCTCATCTCGTCCAGTATGAAC
TfF3H	AB548588.1	CGAGCAGTGGTGAACTCAAACA	TCTGGCGATGGGTTTTGG
TfDFR	AB548587.1	TGGGATGCTTCCGACTTCTG	CAGTGGTTTCTGCCATTGCTT
TfANS	AB548583.1	CCAAGGAGAAGATCGTGCTCA	CAATAAACTCCACCCATCACTCAAC
Tf3GT	AB548591.1	CATTACACACTGCGGTTGGAA	CGGGACACCGCTGCAT
TfF3′5′H	AB548590.1	CTGAACCTGCCTCGGATCTC	CCTCGTGCCCTTTGGTATGT
TfACT3	AB330989.1	TCCCAAAGCCAATCGTGAA	TTGCAGGAGCATTGAAGGTTT

2.4. Statistical Analysis

Data were statistically analyzed using analysis of variance with SPSS version 11.09 (IBM Corporation, Armonk, NY, USA). Data are presented as means ± SE. Groups were compared using Duncan's multiple range test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Sucrose-Induced Anthocyanin Accumulation in WT and Transgenic Plants

Accumulation of anthocyanin was observed in transgenic and WT plants cultured in MS medium containing different concentrations of sucrose (3%, 5%, or 7%). However, accumulation of anthocyanin was significantly higher in transgenic plants compared to WT plants at all sucrose concentrations (Figure 1). Greater anthocyanin accumulation was observed in both WT and transgenic plants at increasingly higher sucrose concentrations (7% > 5% > 3%). Interestingly, greater accumulation of anthocyanin was observed in the RS2 transgenic line (RS2) than the RS1 line, despite the insertion of *RsMYB1* in both lines. Similar results were observed in the BM1 and BM2 transgenic lines overexpressing *B-Peru+mPAP1*, with greater accumulation of anthocyanin observed in the BM1 line compared to the BM2 line. These results indicate sucrose is involved in the regulation of anthocyanin synthesis in a concentration-dependent manner and its effects vary between torenia plant lines.

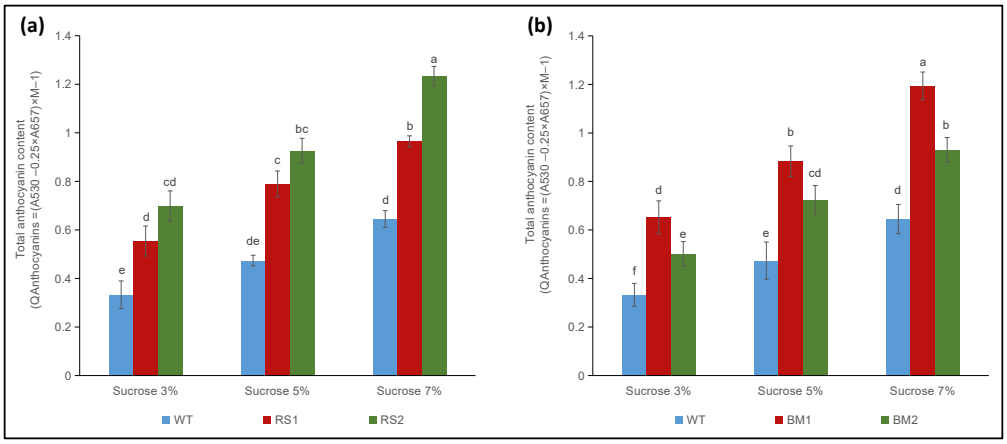

Figure 1. Comparison of total anthocyanin content in transgenic (**a**) *RsMYB1* and (**b**) *B-Peru* + *mPAP1* and wild type (WT) plants of Torenia "Kauai Rose" in response to different sucrose concentrations. Data are presented as the means of three replicates. Error bars indicate standard error. Groups annotated with the same letter are not significantly different by Duncan Multiple Range Test (DMRT, $p < 0.05$).

3.2. Sucrose Regulates Transcription of Anthocyanin Biosynthesis Genes

We evaluated transcript levels of anthocyanin biosynthesis genes (CHS, F3H, F35H, DFR, ANS, and 3GT) using quantitative real time-PCR (qRT-PCR). Differential gene expression was observed according to sucrose concentration and the plant lines. Expression of anthocyanin biosynthesis genes was higher in transgenic plants compared to WT plants at all sucrose concentrations, with greater expression levels observed with greater sucrose concentrations (7% > 5% > 3%). Moreover, anthocyanin biosynthesis gene expression levels were higher in RS2 or BM1 lines compared to RS1 and BM2 lines (Figures 2 and 3). Greater anthocyanin biosynthesis gene expression was associated with greater anthocyanin accumulation. Therefore, these results demonstrate sucrose stimulates anthocyanin accumulation in both WT and transgenic plants by upregulating anthocyanin biosynthesis genes.

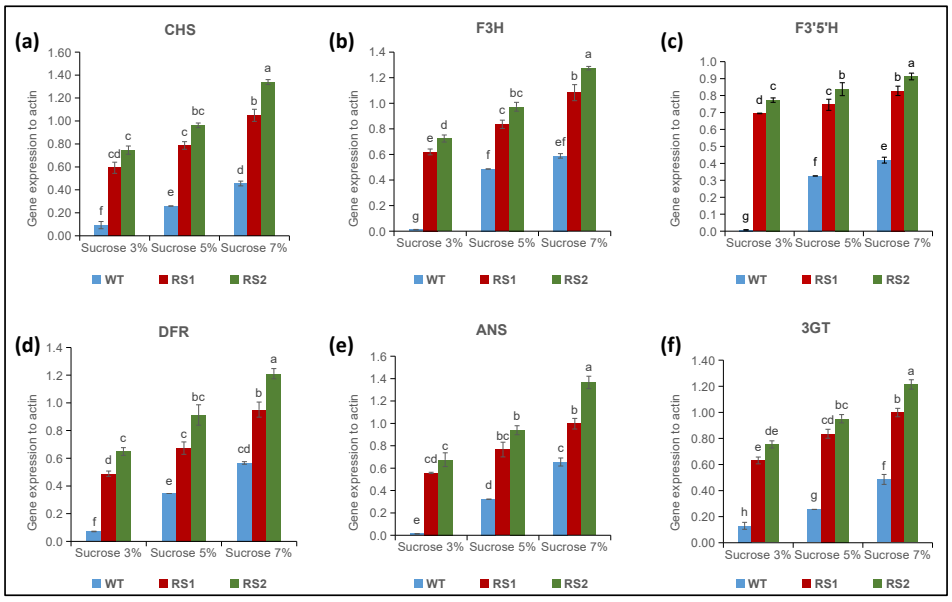

Figure 2. Effect of sucrose on anthocyanin biosynthesis gene expression levels (**a**–**f**) in wild type (WT) plants and transgenic lines overexpressing *RsMYB1*. Data are presented as the means of three replicates. Error bars indicate standard error. Groups annotated with the same letter are not significantly different by Duncan Multiple Range Test (DMRT, $p < 0.05$).

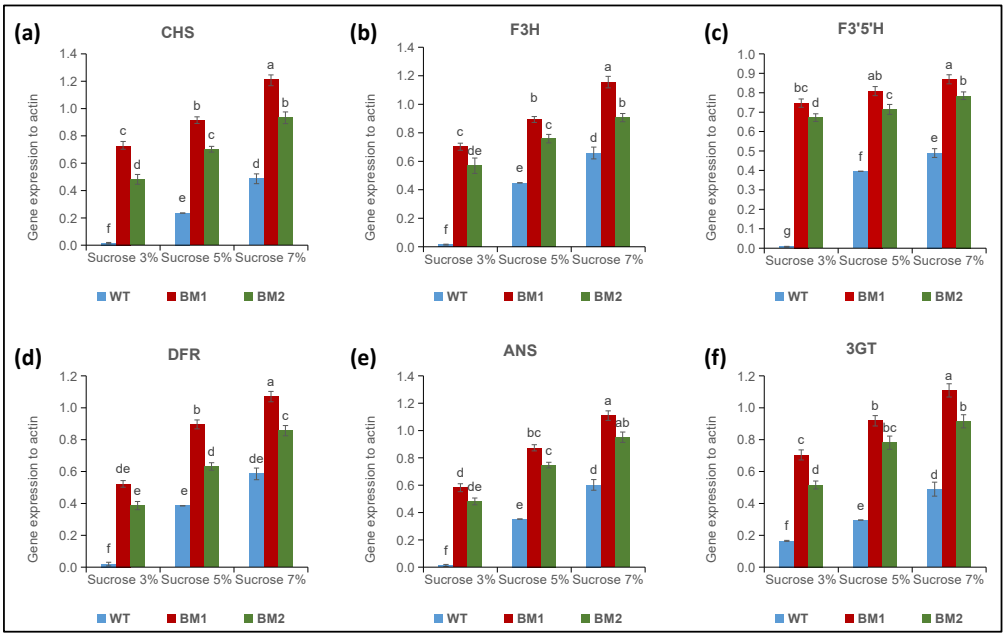

Figure 3. Effect of sucrose on anthocyanin biosynthesis gene expression levels (**a**–**f**) in WT plants and transgenic lines overexpressing *mPAP1* + *B-Peru*. Data are presented as the means of three replicates. Error bars indicate standard error. Groups annotated with the same letter are not significantly different by Duncan Multiple Range Test (DMRT, $p < 0.05$).

3.3. Effect of Sucrose on Plant Growth

Plant growth parameters, including plant height, root length, and fresh weight, were measured at different sucrose concentrations. WT plants cultured in media containing 3% sucrose had greater growth (plant height, root length, and fresh weight) compared to transgenic lines. No significant difference in plant height or root length was observed between WT and RS1 plants. Fresh weight was greatest in WT plants followed by RS1 and RS2 plants (Figure 4). No significant differences in growth parameters were observed between WT and BM lines, other than a significant difference in plant height between WT and BM1 plants (Figure 5). Decreased growth of WT plants and RS lines was observed with 5% sucrose, whereas fresh weight was found to be less reduced in WT plants compared to RS lines (WT > RS1 > RS2). No difference in plant growth was observed between WT plants and BM lines with 5% sucrose. A greater difference in growth between WT plants and RS lines was observed with 7% sucrose, with lower growth in WT plants compared to RS lines. No significant difference in growth was observed between WT and BM lines with 7% sucrose.

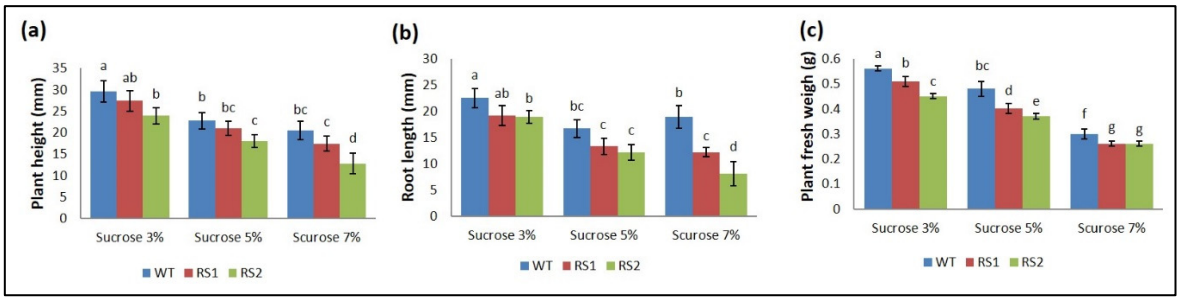

Figure 4. Effect of sucrose on growth attributes ((**a**); plant height, (**b**); root length, (**c**); plant fresh weight) in wild type (WT) plants and transgenic torenia overexpressing *RsMYB1*. Data are presented as the means of three replicates. Error bars indicate standard error. Groups annotated with the same letter are not significantly different by Duncan Multiple Range Test (DMRT, $p < 0.05$).

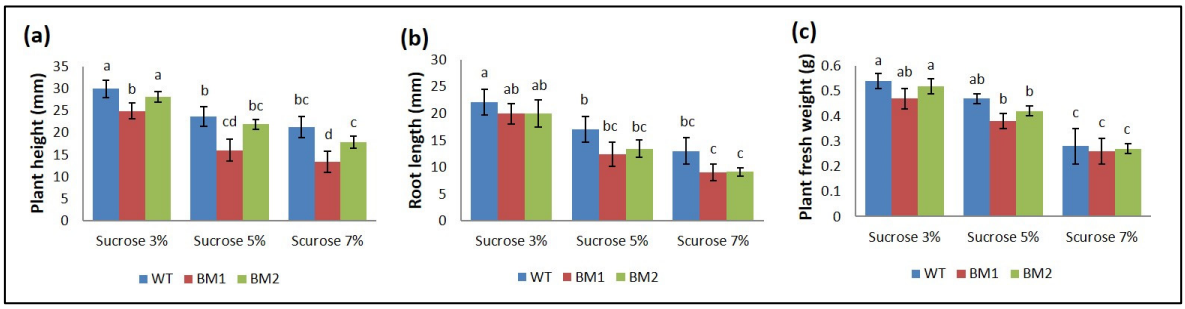

Figure 5. Effect of sucrose on growth attributes ((**a**); plant height, (**b**); root length, (**c**); plant fresh weight) in transgenic torenia overexpressing *mPAP1* + *B-Peru* and wild type (WT) plants. Data represent the means of three replicates. Error bars indicate standard error. Groups annotated with the same letter are not significantly different by Duncan Multiple Range Test (DMRT, $p < 0.05$).

Overall, greater reductions in plant growth were observed with higher concentrations of sucrose in both WT and transgenic lines. However, greater reductions in plant growth were observed in RS lines compared to WT plants, with greater reductions in growth observed in RS2 plants compared to RS1. Plant growth was reduced in BM1 lines compared to WT and BM2 lines.

4. Discussion

Sucrose-induced anthocyanin production via upregulation of EBGs and LBGs has been characterized extensively in vegetative and reproductive tissues of horticultural crops including grape, petunia, and radish [5,6,15–17]. However, the effect of sucrose on anthocyanin production differs according to genotype [7]. Moreover, Ai et al. [18] reported sucrose-induced anthocyanin accumulation in transgenic petunia cv "Mirage Rose" lines overexpressing *RsMYB1* or *B-Peru+mPAP1* but not in WT plants. Therefore, the regulatory mechanisms underlying sucrose-induced anthocyanin biosynthesis remain unclear. While sucrose-induced anthocyanin accumulation has been demonstrated in other torenia cultivars, only higher sucrose concentrations (7%) have been shown to consistently increase anthocyanin accumulation [10,19]. Those studies did not investigate the mechanism by which sucrose regulates anthocyanin biosynthesis. In our previous work, we observed that transgenic torenia plants "Kauai Rose" overexpressing *B-Peru* + *mPAP1* or *RsMYB1* exhibited enhanced anthocyanin accumulation [20]. Therefore, we investigated role of sucrose in anthocyanin synthesis in torenia "Kauai Rose" using both WT and transgenic plants, with a focus on the molecular mechanisms underlying induction of anthocyanin synthesis by sucrose. We further evaluated the effect of sucrose on plant growth and antioxidant activity.

In the present study, anthocyanin accumulation in response to sucrose was observed in both WT and transgenic plants, with greater accumulation seen with increasing concentrations of sucrose (7% > 5% > 3%). These results indicate higher sucrose concentrations had a greater effect on upregulation of anthocyanin biosynthesis genes in both WT and transgenic plants. Results of qRT-PCR analysis demonstrated significantly greater expression levels of anthocyanin biosynthesis genes (*CHS*, *F3H*, *F3′5′H*, *DFR*, *ANS*, and *3GT*) in response to 7% sucrose compared to 5% sucrose, with the lowest expression levels seen with 3% sucrose. These results indicate sucrose stimulates anthocyanin production in both WT and transgenic torenia plants. Our results differ from those of Ai et al. [18] by demonstrating sucrose-induced anthocyanin in transgenic plants overexpressing *B-Peru* + *mPAP1* or *RsMYB1*. In the present study, significantly higher anthocyanin accumulation and anthocyanin biosynthesis gene expression was observed in transgenic plants compared to WT plants. As the *B-Peru* + *mPAP1* and *RsMYB1* insertions in transgenic plants regulate anthocyanin biosynthesis genes, sucrose induces greater expression of anthocyanin biosynthesis genes and production of anthocyanin in transgenic plants compared to WT plants. Previous studies have demonstrated that sucrose upregulates the expression of *CHS*, *DFR*,

and *ANS* genes in grape, petunia, potato, and radish [5,6,15,16,25,26]. In contrast to these previous studies, sucrose promoted expression of *CHS*, *F3H*, *F3′5′H*, *DFR*, *ANS*, and *3GT* in torenia in the present study. Therefore, regulation of sucrose-induced anthocyanin biosynthesis likely differs between plant species. Nagira and Ozeki [10] and Nagira et al. [19] previously reported sucrose dose-dependent anthocyanin accumulation in other torenia cultivars; however, increased expression of anthocyanin biosynthesis genes in response to sucrose was not reported. In transgenic lines expressing *RsMYB1* (RS1 and RS2), greater anthocyanin accumulation is observed in RS2 compared to RS1 in response to different sucrose concentrations, with similar results observed in lines expressing *B-Peru* + *mPAP1* (BM1 > BM2). These differences may be intrinsic to the plant cells from which the transgenic lines were derived or due to greater promoter activity of *RsMYB1* in the RS2 line or of *B-Peru* + *mPAP1* in the BM1 line compared to the RS1 and BM2 lines, respectively.

Sucrose was seen to affect the growth of WT and transgenic plants in the present study, with increased plant height, root length, and fresh weight observed in plants cultured with 3% sucrose compared to 5% and 7% sucrose. Greater growth with 3% sucrose has been reported in previous studies [18,27–29], in which 3% sucrose promoted increased plant growth while greater concentrations reduced plant growth. In the present study, the greater reduction in plant growth observed with media containing higher sucrose concentrations may be due to increased anthocyanin production in response to sucrose, thereby leading to increased degradation of nitrogen and chlorophyll contents resulting in interruption of photosynthesis and plant growth [9,11,13,19,21]. In addition, despite no significant difference in some cases, the greater plant growth observed in WT compared to transgenic plants in response to different sucrose concentrations may be due to increased anthocyanin but lower nitrogen and chlorophyll levels in transgenic plants. Indeed, greater plant growth was observed in RS1 compared to RS2 plants and in BM2 compared to BM1, possibly due to decreased accumulation of anthocyanin in RS1 and BM2 lines compared to RS2 or BM1 lines. Further, the greater osmotic pressure of 5% and 7% sucrose may inhibit nutrient uptake and the development of cultured cells, thereby suppressing plant growth [18,30].

5. Conclusions

The results of the present study demonstrate sucrose stimulates anthocyanin accumulation in both WT and transgenic torenia, with greater accumulation in transgenic plants overexpressing anthocyanin regulatory TFs B-Peru + mPAP1 or RsMYB1. In addition, higher concentrations of sucrose were found to stimulate greater accumulation of anthocyanin in both transgenic and WT plants by promoting increased expression of anthocyanin biosynthetic genes. Sucrose, particularly at higher concentrations, attenuated the growth of WT and transgenic plants. Taken together, these results indicate that sucrose enhances anthocyanin production in torenia by regulating anthocyanin biosynthesis genes.

Author Contributions: A.H.N. designed the experiment. J.X. helped conducting of the experiment. M.Y.C. and K.I.P. assisted the experiments. A.H.N. wrote and revised the manuscript. C.K.K. supervised the project. All authors have read and agreed to the published version of the manuscript.

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