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Short communication

Expression of *RsMYB1* in chrysanthemum regulates key anthocyanin biosynthetic genes

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ABSTRACT

Background: Several *MYB* genes belonging to R2R3 MYB transcription factors have been used in several plant species to enhance anthocyanin production, and have shown various expression or regulation patterns. This study focused on the effect of ectopic expression of an *RsMYB1* isolated from radish (*Raphanus sativa*) on chrysanthemum cv. 'Shinma'.
Results: The RT-PCR results confirmed that *RsMYB1* regulated the expression of three key biosynthetic genes (*CmF3H*, *CmDFR*, and *CmANS*) that are responsible for anthocyanin production in transgenic chrysanthemum, but were not detected in the non-transgenic line. In all transgenic plants, higher expression levels of key biosynthetic genes were observed in flowers than in leaves. However, the presence of *RsMYB1* in chrysanthemum did not affect any morphological characteristics, such as plant height, leaf shape or size, and number of flowers. Furthermore, no anthocyanin accumulation was visually observed in the leaves and floral tissue of any of the transgenic lines, which was further confirmed by anthocyanin content estimation.
Conclusion: To our knowledge, this is the first time the role of an MYB transcription factor in anthocyanin production has been investigated in chrysanthemum.

1. Introduction

It has become popular to use molecular breeding techniques to modify flower color in ornamental flowering crops, and they have been applied to roses, carnations, and chrysanthemums. *Agrobacterium*-mediated genetic transformation is the most commonly used molecular breeding technique for these purposes.

Flower color is determined by the presence of compounds such as flavonoids, carotenoids, and betalains [1]. Anthocyanins belong to the flavonoids and exhibit a wide range of colors. Generally, the three anthocyanin biosynthetic pathways, namely cyanindin, pelargonindin, and delfinindin, which are found in many plants, produce red/pink, brick-red/orange, and blue/violet pigments, respectively [2]. Thus, it seems that at least one of the three biosynthesis pathways is needed to modify flower colors.

MYB plant transcription factors, alone or in combination with basic helix-loop-helix (bHLH) and WD40 transcription factors, have been used to control the expression of genes involved in anthocyanin production [3,4,5,6,7,8,9,10]. Several years ago, the important roles played by *MYB* genes belonging to the R2R3 family in flower color pigmentation were identified [11,12]. In addition, ectopic over-expression of *R2R3 MYB* genes in transgenic plants has also been shown to distinctly enhance anthocyanin accumulation, and in many cases, this affected pigmentation within plant species other than those from which the *MYB* transgenes originated [10,13,14,15,16,17,18,19,20,21]. Ectopic expression of the *Arabidopsis MYB* genes: *AtMYB75* (*PAP1*) and *AtMYB90* (*PAP2*), in *Nicotiana tabacum* enhanced anthocyanin pigmentation in most parts of the transgenic plants [22].

Radish *RsMYB*, which belongs to the R2R3-MYB transcription factor family, was found to be highly expressed in the skin and flesh of three radish cultivars (Seo Ho, Man Tang Hong, and Hong Feng No.1), and causes increased anthocyanin accumulation [23]. Koes et al. [24] also suggested that *RsMYB* might also regulate biosynthetic genes involved in anthocyanin production because it has sequence homology to *Arabidopsis MYB* genes (*PAP1/2*), which are widely known as anthocyanin regulatory genes. According to results from our preliminary experiments, genetic transformation of petunia using *RsMYB1* enhanced anthocyanin production (unpublished data). *InMYB1* encoding the R2R3-MYB transcription factor isolated from morning glory (*Ipomoea nil*), is strongly expressed in the flower tube and limb. Thus, we created the *RsMYB1* construct by placing it under the control of two promoters (a constitutive promoter,

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cauliflower mosaic virus CaMV 35S, and a petal-specific promoter, *InMYB1*, isolated from morning glory). The construct was introduced into commercial chrysanthemum cv. Shinma, which is a hexaploid cultivar and has several desirable horticultural traits, such as large flower size, resistance to rust disease, and a long vase life, but its flower color is white.

Overexpression of *VvMYB5b* occurs in the skin tissue of tomato after fruit color has developed, and this is due to anthocyanin accumulation [20]. The tomato plant showed not only color pigmentation, but also morphological changes, such as dwarfism, and modified leaf structure, flower shape, and fruit texture [25]. Hence, we investigated the morphology of the transgenic plants expressing *RsMYB1*. In addition, since the main anthocyanin biosynthesis pathway in chrysanthemum was the cyanin-based pathway, expressions of three key biosynthetic genes (*CmF3H*. *CmDFR*, and *CmANS*) which are responsible for the anthocyanin pathway were investigated.

2. Materials and methods

2.1. Vector construction and transformation

Agrobacterium tumefaciens strain GV3101, harboring a binary vector, pB7WG2D, and an *RsMYB1* gene isolated from radish (*Raphanus sativa*), was used in this experiment. *RsMYB1* was placed under the control of two promoters, a constitutive promoter (cauliflower mosaic virus CaMV 35S) and a petal-specific promoter (*InMYB1* isolated from morning glory (*I. nil*)), in order to study transformations in chrysanthemum. *Bar* for resistance to PPT was used to select the transgenic plants.

Briefly, 100 leaf explants (about 0.5 cm-long) were incubated with the *A. tumefaciens* strain harboring *RsMYB1*. After co-cultivation at dark condition, the explants were cultured on a medium consisting of MS with 0.5 $mg \cdot L^{-1}$ BA and 0.5 $mg \cdot L^{-1}$ NAA, 3% sucrose, 3 $g \cdot L^{-1}$ gelrite (pH 5.8), and 125 $mg \cdot L^{-1}$ of Clavamox, and placed in the dark at 25°C for 7 d. The explants were then cultured on the same medium, except that it also contained 1.0 $mg \cdot L^{-1}$ of PPT, under a 16 h photoperiod. After 6 weeks, the presence of the selection marker, target, and promoter genes was detected by PCR analysis of the tested samples (data not shown). Three lines (S1, S2, and S3) were confirmed to be transgenic plants, so they were selected for morphological and molecular characterization. The control was a plant regenerated from explants not infected with *Agrobacterium*. The plantlets were then transferred to pots filled with peat soil and placed in the greenhouse.

2.2. Morphological characterization of the transgenic plants

The plant height differences between the transgenic lines and the control plant were considered. In addition, the branching pattern of the plants, flowering time, color pigmentation, and numbers of leaves and flower buds were also compared.

2.3. Isolation of total RNA for reverse transcription polymerase chain reaction (RT-PCR) analysis

The RNA samples were extracted from the leaves, and from flowers at early flowering (F1) and at fully flowering (F2) using an RNeasy® Plant Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. For analysis of *RsMYB1* expression, total RNA extracted from leaves of the three transgenic lines and the non-transgenic line (control) was detected by RT-PCR. In terms of anthocyanin biosynthetic genes expression, the RNA extracted from the leaves, and from flowers at early flowering (F1) and at fully flowering (F2) was detected. The first strand of cDNA was synthesized from 1 μg of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Expression of the *RsMYB1* gene in the samples was first analyzed. The expression levels of the biosynthetic genes (*CmF3H*, *CmDFR*, and *CmANS*) were then normalized using an internal control gene (*CmActin*). PCR primers for the genes are described in Table 1, along with their specific PCR conditions. The biosynthetic gene primers were obtained from the coding sequences of several chrysanthemum plants.

Table 1
Primers and PCR conditions used in this study.

Genes	Primer	PCR conditions
ACTIN	F-ACA ACG TTT TAC AAT GAG CTT CG R-CCG TTC AGC AGT TGT AGT AA	95°C for 2 min, followed by 30 cycles of 95°C for 20 s, 57°C for 40 s, and 72°C for 1 min
F3H	F-ACC CGG TTC GTC CGT GAT GAG G R-TGC CTG GTG GTC CGC ATT CT	95°C for 2 min, followed by 30 cycles of 95°C for 20 s, 63°C for 40 s, and 72°C for 45 s
DFR	F-ATG AAA GAA GAC TCA CCA GCC A R-CTT CGT GAG TGG CCG CCT TT	95°C for 2 min, followed by 30 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 1 min
ANS	F-ATA CAT CCG AAC ACA AGA TG R-AAT CGC TAG GTG TCG AGG GCC	95°C for 2 min, followed by 30 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 30 s
RsMYB1	F-ATG GAG GGT TCG TCC AAA GG R-GAA ACA CTA ATC AAA TTA CAC AGT CTC TCC	98°C for 30 s, followed by undergoing 25 cycles of 98°C for 10 s, and 60°C for 30 s,

2.4. Analysis of anthocyanin content

Total anthocyanin content was analyzed according to the procedure established by Naing et al. [26], with some modification. Briefly, approximately 500 mg of plant material (leaves, or flowers at the early (F1) or fully flowering (F2) stages) was excised from the plants (the three transgenic lines and the control) that had been grown in a greenhouse, and crushed, and the pigments extracted with 5 mL of distilled water. The pigment samples were then incubated overnight at 4°C after 5 mL of a 1% (w/v) hydrochloric acid in methanol solution had been added. The supernatant was collected after centrifugation at 3000 rpm for 20 min and transferred to a 2 mL collection tube. Absorbance was measured at 430–630 nm using a spectrophotometer (U-2800: Hitachi, Tokyo, Japan). The concentration (mg/L) of each sample was calculated according to the report of Sung et al. [27].

3. Results

3.1. Morphological characterization of transgenic lines containing RsMYB1

The presence of *RsMYB1* in the three transgenic lines (S1, S2, and S3) was detected by PCR (data not shown). However, as observed in Fig. 1,

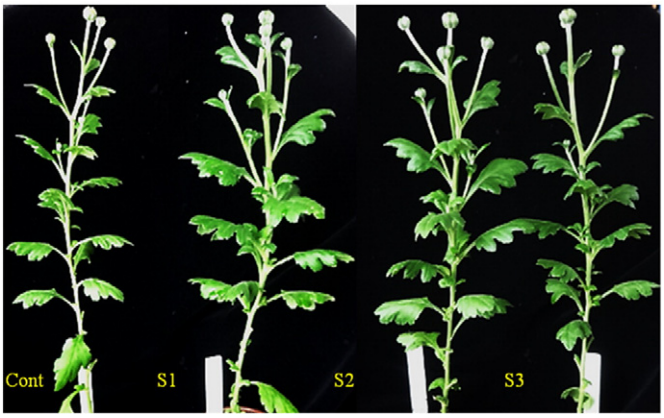

Fig. 1. The non-transgenic (control) and three transgenic lines (S1, S2, and S3) have similar morphologies.

all transgenic lines exhibited similar branching patterns compared to the control, resulting in no morphological differences between the transgenic lines and the control. Plant heights were also similar, with no shortening of the internodes, and there was no large variation in the numbers of leaves and flowers on each plant. The flowering time of the transgenic plants was found to be the same as that of the control plants, and there were no differences in leaf size and shape. Although the presence of *RsMYB1* gene was detected by PCR analysis (data not shown), this analysis did not show the exact gene expressions because gene silencing and methylation were reported in several plant transformations. Thus, confirmation of *RsMYB1* expression by RT-PCR was performed, and it showed that the expression levels of *RsMYB1* in the transgenic lines were stable (Fig. 2). Surprisingly, no difference in color pigmentation was observed in either the leaves or flowers compared to the control plant (Fig. 3), even though *RsMYB1* was distinctly expressed.

3.2. RsMYB1 gene regulates the biosynthetic genes in chrysanthemum

The expression of the three biosynthetic genes (*CmF3H*, *CmDFR*, and *CmANS*) was analyzed by RT-PCR to verify whether *RsMYB1* regulated the biosynthetic genes responsible for the anthocyanin biosynthetic pathway. As Fig. 4 shows, *CmF3H* was expressed in all parts of the transgenic plants, whereas in the control, *CmF3H* was only expressed in the flowers at the early flowering stage (F1), and not in the leaves (L) or flowers at the fully flowering stage (F2). Moreover, *CmDFR* was not expressed at all in the control, whereas it was expressed in the transgenic lines. Interestingly, *CmANS* could be detected in both the control and the transgenic lines. *CmF3H* showed higher transcript levels in flowers at the fully flowering stage, followed by the early flowering stage, but much lower levels in the leaves of the transgenic plants, whereas in the control, it was only expressed in flowers at the F1 stage. Expression of *CmDFR* was stable in all tested parts of the transgenic lines, but was downregulated as compared to *CmActin* (internal control). *CmANS* expression level was similar in all tested parts of the control plants; however, *CmANS* was differentially expressed in the transgenic lines. The expression levels were generally higher in flowers than in the leaves of the transgenic plants. Overall, the RT-PCR analysis showed that the three key structural genes responsible for anthocyanin accumulation were expressed in all tested parts of the transgenic lines, but only in certain parts of the control plant.

No anthocyanin accumulation was detected by morphological observations. However, expression of the three key structural genes could be detected; hence it was assumed that anthocyanin had accumulated inside the leaves or flowers without it being expressed morphologically. Thus, anthocyanin was extracted from the transgenic leaves and flowers at the two different flowering stages (F1 and F2) and compared to extracts from the control plant. Fig. 5 shows that there was no color difference, which agreed with the anthocyanin results for all tested parts of the transgenic and control plants. It was further confirmed by a spectrophotometer that anthocyanin levels were very low (approximately 0.001 mg g^{-1}) in all the tested samples (Fig. 6).

4. Discussion

To date, genetic transformation techniques have been used to develop transgenic chrysanthemums [28,29,30,31,32]. However, transgenic chrysanthemums containing foreign *MYB* have not been

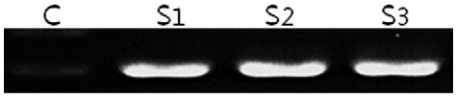

Fig. 2. RT-PCR analysis of *RsMYB1* expression in the non-transgenic line (control, C) and the three transgenic lines (S1, S2, and S3).

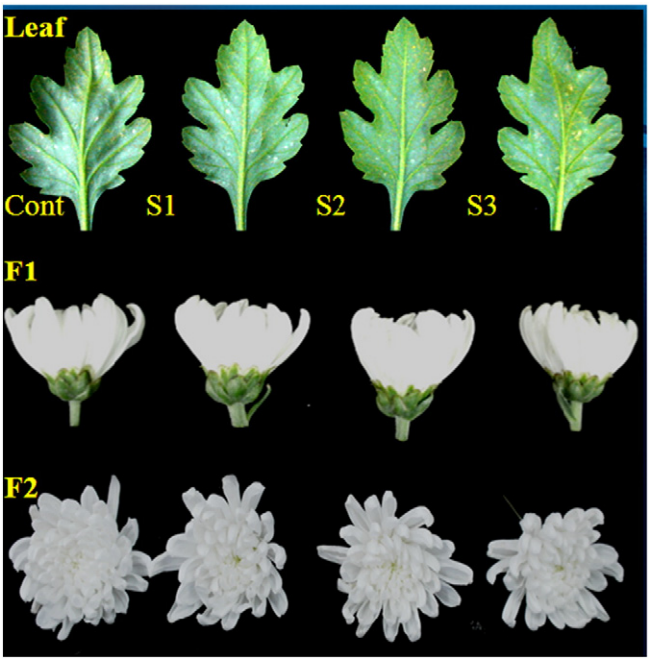

Fig. 3. Morphological characterization of the leaf and the two different flower stages of the non-transgenic (control, cont) and the three transgenic lines (S1, S2, and, S3) containing *RsMYB1*. L: leaf. F1: early flowering stage, and F2: fully flowering stage.

developed so far. In general, the introduction of MYB transcription factors in many different plants has been shown to enhance color pigmentation. However, it has affected the morphology of transgenic plants. Mahjoub et al. [25] reported that when *Vitis vinifera* R2R3-MYB transcription factor (coded by *VvMYB5b*) was over expressed in tomato, there were color pigmentation changes and morphological variations, such as dwarfism, modified leaf structure, flower shape, and fruit texture. *VvMYB5b* was highly expressed in the skin tissue of the fruit after the fruit had begun to change color [20] and the fruit color change strongly enhanced anthocyanin accumulation. However, in this study, *RsMYB1* cDNA, which is strongly expressed in the skin and flesh of radish and leads to anthocyanin accumulation, produced no phenotypic changes when expressed in chrysanthemum.

Transcription factors increase the amount of anthocyanins in many plant species. MYC and MYB transcription factors regulate the expression of biosynthetic genes in maize. Similar transcription factors in snapdragon and petunia also regulate the expression of biosynthetic genes [33,34]. Ectopic expression of *Del* encoding MYC transcription factor from snapdragon in tobacco has led to increased anthocyanin accumulation when driven by the CaMV 35S promoter [26]. The *MYC* (*Lc*) transcription factor gene from maize, controlled by the same CaMV 35S promoter, changed flower color from pink to intense red in tobacco, and enhanced anthocyanin production in all vegetative tissues in tomato [35], and in both vegetative and floral tissues of petunia [36]. However, some transgenic plants, such as chrysanthemum, rose, and carnation, wherein maize *Lc* had been introduced, did not show a significant increase or decrease in anthocyanin accumulation [37, 38]. Likewise, MYB transcription factor modified the color of white flowers of *Petunia axillaris* and purple flowers of *Petunia integrifolia* [39]. However, it showed different pigmentation control activities in different species of *Antirrhinum* and *Petunia* [39], and had different pigmentation effects on berry skin color in grape [40,41] and on tuber color in potato [28]. There seems to be some variation in the ability of transcription factor activity to control anthocyanin production under ectopic conditions.

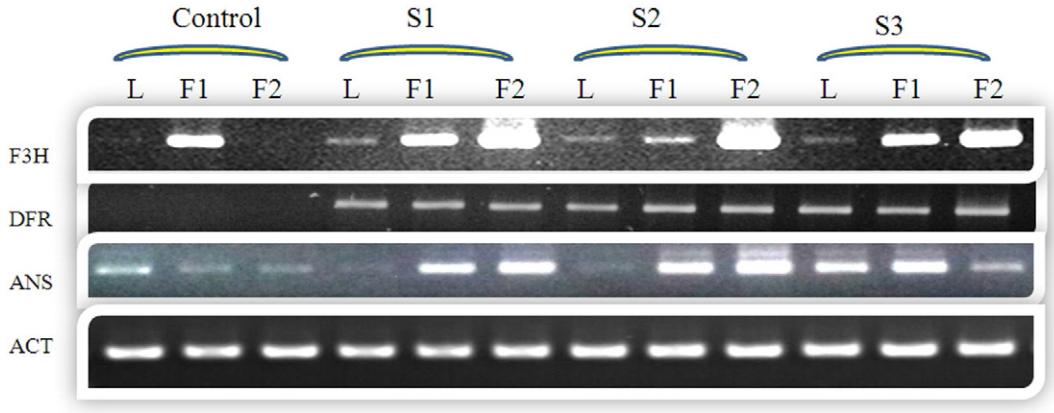

Fig. 4. RT-PCR analysis of the expression patterns of biosynthetic genes in the leaf and at the two different flower stages for the non-transgenic (control, cont) and the three transgenic lines (S1, S2, and, S3) containing *RsMYB1*. L: leaf. F1: early flowering stage, and F2: fully flowering stage.

In this study, three transgenic chrysanthemum plants expressing radish *RsMYB1* cDNA, which was driven by CaMV 35S and the petal specific promoter, *InMYB1*, were obtained. Interestingly, no pigmentation was detected in either vegetative or floral organs. The RT-PCR results show that the *RsMYB1* cDNA fragment could activate expression of the three anthocyanin biosynthetic genes, *CmF3H*, *CmDFR*, and *CmANS*, which are responsible for anthocyanin biosynthesis, and that the expression levels of *CmF3H* and *CmANSm* were generally higher in floral tissue than in leaves. This might be attributed to the activity of the *InMYB1* promoter. In contrast, *CmDFR* was stably expressed in all tested parts of the transgenic plants. As *RsMYB1* was able to change color pigmentation in petunia according to our preliminary experiment (data not shown), the gene construct was used for chrysanthemum transformation. Even though *RsMYB1* could regulate the three key biosynthetic genes involved in the anthocyanin biosynthetic pathway in chrysanthemum, the reason why anthocyanin expression was not observed in any of the chrysanthemum transgenic lines is not clear. One possible explanation is that 'Shinma' does not have an anthocyanin biosynthetic pathway, even though *in vitro* plantlets of 'Shinma' showed occasional anthocyanin pigmentation in their axial parts (data not shown). Alternatively, the anthocyanin biosynthetic pathway may contain a different set of structure genes. These contradictory results need to be resolved by future studies on chrysanthemum transgenic lines.

Conflict of interests

The authors declare that there is no conflict of interest

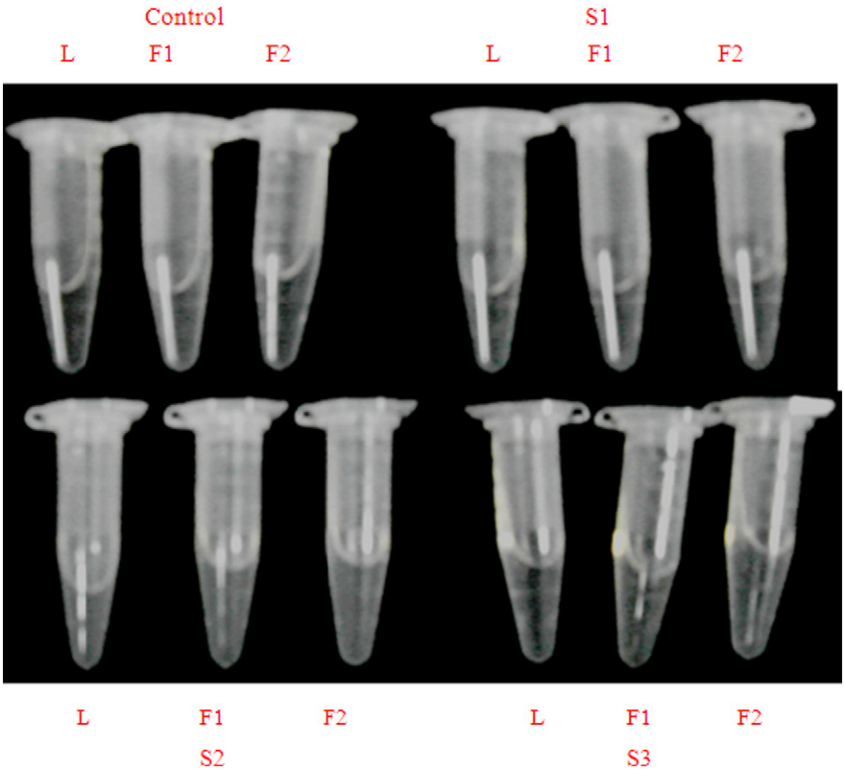

Fig. 5. Comparative analysis of anthocyanin content between the non-transgenic (control) and the *RsMYB1*-transformed cv. 'Shinma' lines S1, S2, and S3. L: leaf. F1: early flowering stage, and F2: fully flowering stage.

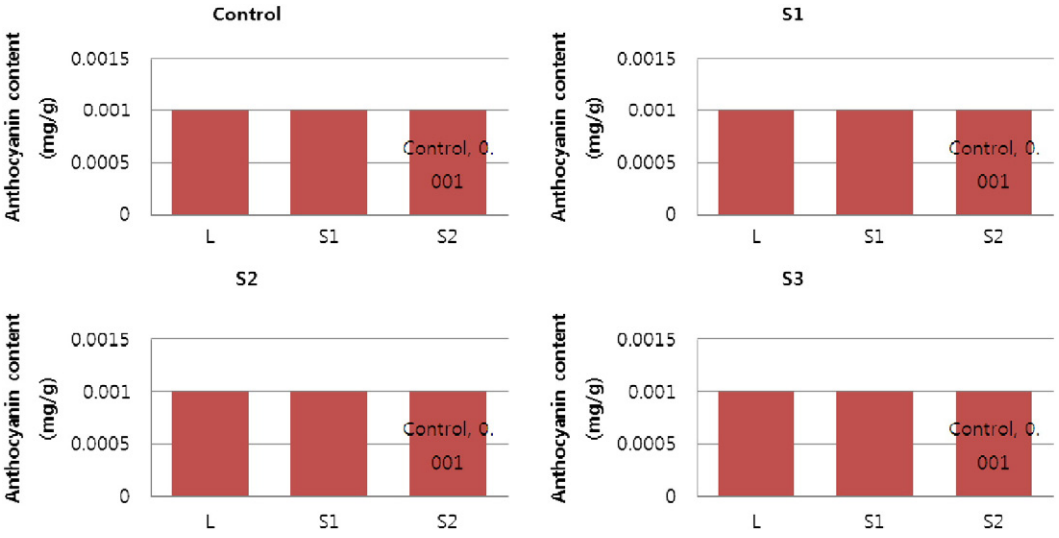

Fig. 6. Spectrophotometric analysis of anthocyanin content between the non-transgenic (control) and the *RsMYB1*-transformed cv. 'Shinma' lines S1, S2, and S3. L: leaf. F1: early flowering stage, and F2: fully flowering stage.

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