Plant Physiology and Biochemistry 89 (2015) 1–10

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Heterologous expression of *IbMYB1a* by different promoters exhibits different patterns of anthocyanin accumulation in tobacco

Chul Han An [a, c, 1], Ki-Won Lee [d, 1], Sang-Hoon Lee [d], Yu Jeong Jeong [a], Su Gyoung Woo [a], Hyokon Chun [a], Youn-Il Park [c], Sang-Soo Kwak [b], Cha Young Kim [a, *]

[a] Eco-friendly Bio-Material Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 580-185, Republic of Korea
[b] Plant Systems Engineering Research Center, KRIBB, Daejeon 305-806, Republic of Korea
[c] Department of Bioscience and Biotechnology, Chungnam National University, Daejeon 305-806, Republic of Korea
[d] Grassland and Forages Division, National Institute of Animal Science, Rural Development Administration, Cheonan 331-801, Republic of Korea

ARTICLE INFO

Article history:
Received 25 November 2014
Accepted 5 February 2015
Available online 7 February 2015

Keywords:
Sweetpotato
Anthocyanin
R2R3-MYB transcription factor
Transgenic plants
Promoter
Tobacco

ABSTRACT

We previously reported that the transient and stable expression of *IbMYB1a* produced anthocyanin pigmentation in tobacco leaves and transgenic *Arabidopsis* plants, respectively. To further determine the effects of different promoters on the expression of *IbMYB1a* and anthocyanin production, we generated and characterized stably transformed tobacco (*Nicotiana tabacum* SR1) plants expressing *IbMYB1a* under the control of three different promoters. We compared the differences in anthocyanin accumulation patterns and phenotypic features of the leaves of these transgenic tobacco plants during growth. Expression of *IbMYB1a* under the control of these three different promoters led to a remarkable variation in anthocyanin pigmentation in tobacco leaves. The anthocyanin contents of the leaves of the *SPO-IbMYB1a*-OX (SPO-M) line were higher than those of the *SWPA2-IbMYB1a*-OX (SPA-M) and *35S-IbMYB1a*-OX (35S-M) lines. High levels of anthocyanin pigments negatively affected plant growth in the SPO-M lines, resulting delayed growth and, occasionally, a stunted phenotype. Furthermore, HPLC analysis revealed that transcriptional regulation of *IbMYB1a* led to the production of cyanidin-based anthocyanins in the tobacco plants. In addition, RT-PCR analysis revealed that *IbMYB1a* expression induced the up-regulation of several structural genes in the anthocyanin biosynthetic pathway, including DFR and *ANS*. Differential expression levels of *IbMYB1a* under the control of different promoters were highly correlated with the expression levels of the structural genes, thereby affecting anthocyanin production levels. These results indicate that *IbMYB1a* positively controls the expression of multiple anthocyanin biosynthetic genes and anthocyanin accumulation in heterologous tobacco plants.

1. Introduction

Plants synthesize tens of thousands of secondary metabolites, such as phenylpropanoids, flavonoids, terpenoids, glucosinolates, and alkaloids (Stitt et al., 2010). Anthocyanins, the largest subclass of flavonoids, confer the purple, red, and blue colors present in plants (Holton and Cornish, 1995; Tanaka et al., 2008). These secondary metabolites play important roles in various plant functions, such as pigmentation and, phytoprotection against damage by ultraviolet light and pathogens (Holton and Cornish, 1995; Winkel-Shirley, 2001). Anthocyanins are important plant pigments with excellent antioxidant properties, thus providing nutritional value for consumers and serving as a possible source of natural food colorants (Nagata et al., 2003). Sweetpotato (*Ipomoea batatas* [L.] Lam) is the seventh most

Abbreviations: CaMV, cauliflower mosaic virus; SPO, sporamin; SWPA2, sweetpotato anionic peroxidase2; bHLH, basic helix-loop-helix; PAP1, Production of Anthocyanin Pigment1; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; HPLC, high-performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction; DPPH, 2,2-diphenyl-1-picrylhydrazyl; APX, ascorbate peroxidase; CAT, catalase.

* Corresponding author. Eco-friendly Bio-Material Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 181 Ipsin-gil, Jeongeup-si, Jeonbuk 580-185, Republic of Korea.
E-mail address: kimcy@kribb.re.kr (C.Y. Kim).
[1] These authors contributed to the work equally.

http://dx.doi.org/10.1016/j.plaphy.2015.02.002
0981-9428/

important food crop worldwide (Rodriguez-Bonila et al., 2014). Sweetpotatoes are rich in secondary metabolites, especially antioxidant compounds, including carotenoids, anthocyanins, and vitamin C (Teow et al., 2007). Purple-fleshed sweetpotato provides a healthy food source for humans and serves as a potential source of natural food colorants due to its high levels of anthocyanins and starch.

Anthocyanin biosynthesis and its regulation have been well characterized in several model plants, such as maize, petunia, and *Arabidopsis* (Bartel and Matsuda, 2003; Holton and Cornish, 1995). Anthocyanin biosynthesis is genetically determined by structural and regulatory genes. A large number of studies have suggested that anthocyanin biosynthesis is controlled by numerous regulatory factors at the transcriptional level, which modulate the expression of structural genes encoding both early- and late-acting enzymes in the anthocyanin biosynthetic pathway (Petroni and Tonelli, 2011). Among the various regulatory proteins, such as MYB, bHLH, and WD40 proteins, MYB transcription factors (TFs) are the major determinants of anthocyanin production (Allan et al., 2008; Hichri et al., 2011). Two redundant MYB TFs, Production of Anthocyanin Pigment1 (PAP1) and PAP2 in *Arabidopsis* and their orthologs in other plant species, are positive regulators of the structural genes involved in anthocyanin biosynthesis, including CHS, DFR, and LDOX (Borevitz et al., 2000; Hichri et al., 2011). Overexpression of *PAP1*/*AtMYB75* in transgenic *Arabidopsis* induces anthocyanin accumulation by increasing the expression of structural genes in the anthocyanin biosynthetic pathway (Borevitz et al., 2000; Dubos et al., 2010; Tohge et al., 2005). In grape, the expression of *VvMYBA1* and *VvMYBA2* leads to the transcriptional activation of the UDP-Glc:flavonoid 3-*O*-glucosyltransferase (*UFGT*) gene as well as anthocyanin biosynthesis (Cutanda-Perez et al., 2009; Geekiyanage et al., 2007; Kobayashi et al., 2002; Li et al., 2011; Walker et al., 2007). In addition, ectopic expression of *VvMYB5b* in tobacco leads to the up-regulation of most flavonoid structural genes, including CHS, CHI, F3H, and ANS, resulting in the accumulation of anthocyanidin- and proanthocyanidin-derived compounds in flowers (Deluc et al., 2008). In apple fruit, *MdMYB10* controls red flesh coloration, while *MdMYB1*, *MdMYBA*, and *MdMYB3* are responsible for red skin coloration (Ban et al., 2007; Espley et al., 2007; Vimolmangkang et al., 2013). Furthermore, overexpression of *MdMYB3* in transgenic tobacco plants results in transcriptional activation of several flavonoid pathway genes, including CHS, CHI, UFGT, and FLS, these plants exhibit increased pigmentation and accumulate higher levels of anthocyanins and flavonols in flowers, but not in leaves (Vimolmangkang et al., 2013). Likewise, in the purple-fleshed sweetpotato, *IbMYB1* is responsible for purple pigmentation in the flesh of storage roots (Mano et al., 2007). Two transcript variants of *IbMYB1a* and *IbMYB1b* appear to be produced from the single *IbMYB1* gene by alternative splicing in purple-fleshed sweetpotato (Kim et al., 2010; Mano et al., 2007). Recently, Park et al. (2015) reported that overexpression of *IbMYB1a* in a single orange-fleshed sweetpotato cultivar generated dual-pigmented transgenic plants with high levels of both anthocyanins and carotenoids in their storage roots. In addition, the expression of *IbMYB1a* alone was sufficient for heterologous activation of anthocyanin biosynthesis in transiently agro-infiltrated tobacco leaves and stably transformed *Arabidopsis* plants (Chu et al., 2013; Kim et al., 2010; Mano et al., 2007). Identifying the interacting partners of *IbMYB1a* TF is necessary for elucidating the gene regulatory networks controlling anthocyanin biosynthesis in purple-fleshed sweetpotato.

Numerous studies have demonstrated that R2R3-MYB TFs in various plant species are primarily responsible for the regulation of anthocyanin biosynthetic pathways. Thus, understanding the regulatory roles of R2R3-MYB TFs in anthocyanin biosynthesis is essential for performing metabolic engineering of the anthocyanin biosynthetic pathway in food crops. In this respect, R2R3-MYB TFs appear to be key factors for metabolic engineering of the anthocyanin biosynthetic pathway to produce altered anthocyanin profile and, hence, altered pigmentation of leaves, flowers, and roots.

In this study, we demonstrated that heterologous expression of the sweetpotato *R2R3-MYB* gene, *IbMYB1a*, under the control of three different promoters produced different anthocyanin levels and accumulation patterns in the leaves of transgenic tobacco plants. Expression of *IbMYB1a* induced anthocyanin accumulation in all tobacco tissues, including leaves, stems, roots, and flowers. In addition, the level of anthocyanin accumulation in transgenic tobacco plants was positively correlated with the antioxidant activities of DPPH, APX, and CAT.

2. Materials and methods

2.1. Plant materials and growth conditions

Surface-sterilized seeds of axenic tobacco plants (*Nicotiana tabacum* SR1) were germinated under aseptic conditions on solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose (pH 5.8) and 0.8% agar (Duchefa). The plates were incubated under long-day growth conditions (16 h light/8 h dark cycle) at 25 °C for germination, and the plants were grown under the same light and temperature conditions. Leaf discs approximately 10 mm in length from 3- to 4-week-old healthy leaves were used for plant transformation.

2.2. Vector construction and plant transformation

Sweetpotato *IbMYB1a* cDNA was cloned into the pCambia2300 binary vector as described by Kim et al. (2010). The resulting pCam2300-IbMYB1a constructs containing different promoters, designated pCam-35S-IbMYB1a, pCam-SWPA2-IbMYB1a, and pCam-SPO-IbMYB1a, were introduced into *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium* cells were grown overnight at 28 °C in YEP medium (10 g/l Bacto peptone, 10 g/l Bacto yeast extract, and 5 g/l NaCl, pH 7.0) containing 100 mg/l kanamycin and 40 mg/l rifampicin. Tobacco was transformed with LBA4404 using the leaf disc method (Horsch and Klee, 1986). *Agrobacterium* cells were grown to an optical density of 1.0 at 600 nm (OD_{600}), and a final suspension at OD_{600} of 0.5 was used for plant infection. Young, healthy green leaves were cut into pieces approximately 10 mm in length, and the leaf segments were incubated in an Agrobacterium suspension for 30 min. The leaf segments were then blotted dry with sterile filter paper for 5 min and placed onto MS1 co-cultivation medium (MS + 0.1 mg/l NAA + 1 mg/l BA) in sterile Petri dishes. After incubation for 3 days at 25 °C in the dark, the infected leaf explants were transferred to MS2 regeneration/selection medium (MS1 + 200 mg/l kanamycin + 250 mg/l cefotaxime). After 2–3 weeks, the infected leaf explants were transferred to fresh regeneration/selection media. Separate shoots from explants were excised carefully and transferred onto plant culture dishes containing MS3 rooting medium (MS + 200 mg/l kanamycin + 250 mg/l cefotaxime). All media were supplemented with 3% sucrose and 0.4% Gelrite (Duchefa), and the pH was adjusted to 5.8. The conditions used for shooting and rooting were the same as those used for germination. Healthy seedlings were transplanted into pots in a growth chamber (25 °C, 16 h light/8 h dark cycle).

2.3. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Two leaf tissues from each of the two independent tobacco lines were collected and ground in liquid nitrogen. Total RNA was extracted from the frozen samples (approximately 0.1 g) using FavorPrep™ Tri-RNA reagent (Favorgen), and further purified using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of RNA was quantified at 260 nm by spectrophotometric measurement. First-strand cDNAs were synthesized from 2 μg of total RNA using a RevertAid™ first-strand cDNA synthesis kit (Fermentas). RT-PCR was performed using Ex-Taq DNA polymerase (TaKaRa) as described by Chu et al. (2013). Control RT-PCR was performed using a primer pair specific to the *ACTIN* gene under the same conditions. Then, 10-μl aliquots of each RT-PCR product were analyzed on a 1.0% agarose gel to visualize the amplified cDNAs. The primers used in this study are shown in Supplementary Table 1.

2.4. Extraction of anthocyanins and measurement of anthocyanin contents by spectrophotometry

Two leaf tissue samples from each of the two independent tobacco lines were collected and ground in liquid nitrogen. Total anthocyanins were extracted from 0.1 g finely ground plant material and measured spectrophotometrically at wavelength 530 nm and 657 nm as described by Chu et al. (2013). All samples were measured in triplicates with two independent biological replicates. Error bars indicate the standard deviation (SD) of the average anthocyanin content.

2.5. Hydrolysis of anthocyanins and HPLC analysis of anthocyanidin profiles

Anthocyanins were further hydrolyzed by boiling in butanol:HCl solvent as described by Chu et al. (2013), and the anthocyanin profiles of tobacco leaf samples were analyzed by HPLC. HPLC analysis was performed at 37 °C on an Agilent 1200 system (Agilent Technologies) equipped with a binary pump, an in-line degasser, an auto-sampler, and a UV detector using a silica C18 reverse phase column (GEMINI 5 μm C18 column, 4.6 × 150 mm, 5 μm; Phenommenex) with a linear gradient of the binary solvent system. The binary solvent system comprised with solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). The gradient was applied linearly from 85:15 (A:B, v/v) to 5:95 (A:B, v/v) for 50 min with a flow rate of 0.5 ml/min. The injection volume was 10 μl and the detection wavelength was set to 530 nm. Cyanidin, delphinidin, peonidin, and malvidin standards (Sigma–Aldrich) were used for identification and quantification.

2.6. Antioxidant enzyme extraction and activity assays

Two leaf tissue samples from each of the two independent tobacco lines were collected and ground in liquid nitrogen, and 0.1 g finely ground leaf tissue samples were used for antioxidant enzyme assays. Total phenolic and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays were spectrophotometrically performed (Ainsworth and Gillespie, 2007; Sharma and Bhat, 2009). For the total phenolic content, leaf samples (0.1 g) were extracted with 0.5 ml of 80% (v/v) ethanol. After centrifugation, 10 μl of the supernatant was mixed with 890 μl of 0.1 N Folin-Ciocalteu reagent and 100 μl of 20% (w/v) Na_2CO_3 was added. The mixture was incubated at room temperature for 20 min and the absorbance was determined at 725 nm using a UV–visible spectrophotometer. Total phenolics were calculated as chlorogenic acid equivalents using the regression equation between chlorogenic acid standards and absorbance at 725 nm. For DPPH assay, leaf samples (0.1 g) were extracted with 0.5 ml of 95% (v/v) ethanol. After centrifugation, 25 μl of the supernatant was mixed with 975 μl of 0.15 mM DPPH solution. The mixture was incubated at room temperature for 30 min and the absorbance was determined at 517 nm using a UV–visible spectrophotometer. L-Ascorbic acid (AsA) was used as a standard for the calibration curve and the DPPH radical scavenging activities were calculated as AsA equivalents per gram of tested samples. For antioxidant enzyme activity assays of ascorbate peroxidase (APX) and catalase (CAT), total soluble proteins were extracted from 0.1 g leaf samples with extraction buffer (APX, 50 mM HEPES, pH 7.0 + 0.1 mM EDTA; CAT, 50 mM potassium phosphate, pH 7.0). APX activity was determined from the decrease in absorbance at 290 nm for 1.5 min caused by ascorbic acid oxidation according to Nakano and Asada (1981). The reaction mixture contained 50 mM HEPES (pH 7.0), 0.6 mM ascorbate, 1 mM H_2O_2, 0.1 mM EDTA and protein extract. One unit of APX activity was defined as the amount of enzyme that caused the oxidation of 1 μmol of AsA. CAT activity was measured spectrophotometrically according to the method of Aebi (1984) by following the consumption of H_2O_2 at 240 nm for 1 min in 50 mM potassium phosphate (pH 9.0) containing 20 mM H_2O_2 and protein extract in a final volume of 1 ml. Reaction was started by adding H_2O_2. One unit of CAT activity was defined as 1 μmol of H_2O_2 consumption per minute and the specific activity was represented as units per mg protein.

2.7. Statistical analysis

Experimental data were analyzed by one-way ANOVA. Subsequent multiple comparisons were examined by using Dunnett test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS version 12) software and statistical significance was set at $^{*}P < 0.05$ and $^{**}P < 0.01$.

3. Results

3.1. Generation of transgenic tobacco plants carrying IbMYB1a driven by three different promoters

We previously reported that both transient expression of sweetpotato *IbMYB1a* in tobacco leaves and stable expression of this gene in *Arabidopsis* plants induce anthocyanin accumulation (Chu et al., 2013; Kim et al., 2010). In this study, to further examine whether *IbMYB1a* acts as a positive regulator of anthocyanin biosynthesis in tobacco plants, we transferred *IbMYB1a* gene into tobacco through *Agrobacterium*-mediated leaf disc transformation. Transgenic tobacco plants expressing *IbMYB1a* were generated under the control of three different promoters, including the duplicated *CaMV 35S* (*d35S*) promoter, the sporamin *SPO* promoter, and the peroxidase *SWPA2* promoter derived from sweetpotato (Fig. 1). The transgenic status and expression of the transgene in transgenic plants were confirmed by selection with the antibiotic kanamycin and visible anthocyanin color selection followed by PCR. Some calli induced from the infected leaf discs exhibited the purple anthocyanin color. Putative transgenic shoots from purple calli also exhibited visible anthocyanin coloration (Fig. 1B). We then transferred healthy purple shoots to root induction medium to generate putative transgenic plants. Using PCR analysis, we confirmed that all of the putative purple seedlings showing visible anthocyanin coloration were transgenic plants (data not shown). This result indicates that transgenic plants harboring the *IbMYB1a* gene could easily be selected through visible color selection without performing PCR analysis. Ten independent lines (T0 generation)

harboring *IbMYB1a* were ultimately obtained and allowed to set seed. Nine and six of ten T0 35S-M and SPA-M transgenic lines produced seeds, respectively, whereas seven of ten SPO-M lines failed to set seed. In addition, we observed differential anthocyanin accumulation patterns in leaves among lines harboring the three constructs (Fig. 1C). SPO-M lines displayed higher levels of anthocyanin and accumulated even anthocyanin pigmentation throughout their leaves than lines harboring the two constructs. However, plant growth in the SPO-M lines occurred relatively late during the growth stage. These findings suggest that high levels of anthocyanin accumulation have a deleterious effect on plant growth and seed development.

3.2. Different levels and patterns of anthocyanin accumulation in transgenic tobacco plants

To further examine the differences in anthocyanin accumulation levels and patterns in the leaves among the three types of transgenic lines, we obtained T1 generation seeds from each T0 transgenic line and observed the phenotypic features of the T1 plants during 3 months of plant growth. T1 plants were selected for antibiotic resistance by germinating the seeds on MS medium supplemented with kanamycin, followed by transfer to soil. The plants were grown to maturity in a growth chamber for 3 months. The appearance of the anthocyanin pigmentation in transgenic leaves varied among the three lines depending on the promoter used. As shown in Fig. 2, at 14 days of seedlings growth on MS medium, all T1 transgenic lines exhibited anthocyanin pigmentation in their cotyledons and leaves but not in roots. Relatively low levels of anthocyanin accumulated in the 35S-M and SPA-M lines, whereas the SPO-M line exhibited high levels of anthocyanin pigmentation in both cotyledons and leaves. The SPA-M line accumulated more anthocyanin in its cotyledons than in its leaves. There were apparent differences in anthocyanin accumulation levels and patterns among the three transgenic lines when grown in soil, as observed in the plants at 28 days and 50 days after germination. There were significant differences in anthocyanin contents and distribution patterns in the leaf tissues among the transgenic lines expressing *IbMYB1a* under the control of different promoters. As shown in Fig. 2, anthocyanin accumulation was restricted to certain areas in the leaves of the 35S-M line and scattered randomly throughout the leaf tissue in the SPA-M line, while the SPO-M line uniformly and highly accumulated anthocyanin pigments throughout the surface area of the leaf tissue. However, the SPO-M line exhibited slow growth, short internodes, and delayed plant growth, although normal growth occurred at the late growth stage. Occasionally, the growth of SPO-M plants exhibiting strong anthocyanin accumulation was stunted, and the plants did not flower or set seed. In addition, the flowers of the transgenic lines were red, unlike the pink color observed in wild-type (WT) tobacco plants. The flowers of the 35S-M and SPO-M lines had dark red petals and sepals, and the SPA-M line had red flower petals and sepals. By contrast, the sepals of WT flowers were green.

3.3. Expression of IbMYB1a leads to the production of cyanidin-based anthocyanins in transgenic tobacco plants

To examine whether the expression of *IbMYB1a* in transgenic tobacco plants led to the production of anthocyanins, we examined the total anthocyanin contents in 50-day-old leaf tissues in all three types of *IbMYB1a*-OX lines (T1) using spectrometric measurements (Fig. 3A). The highest levels of total anthocyanins were observed in the leaves of the SPO-M line, with values of approximately 1.1 mg of anthocyanin per gram fresh weight, which represented an almost

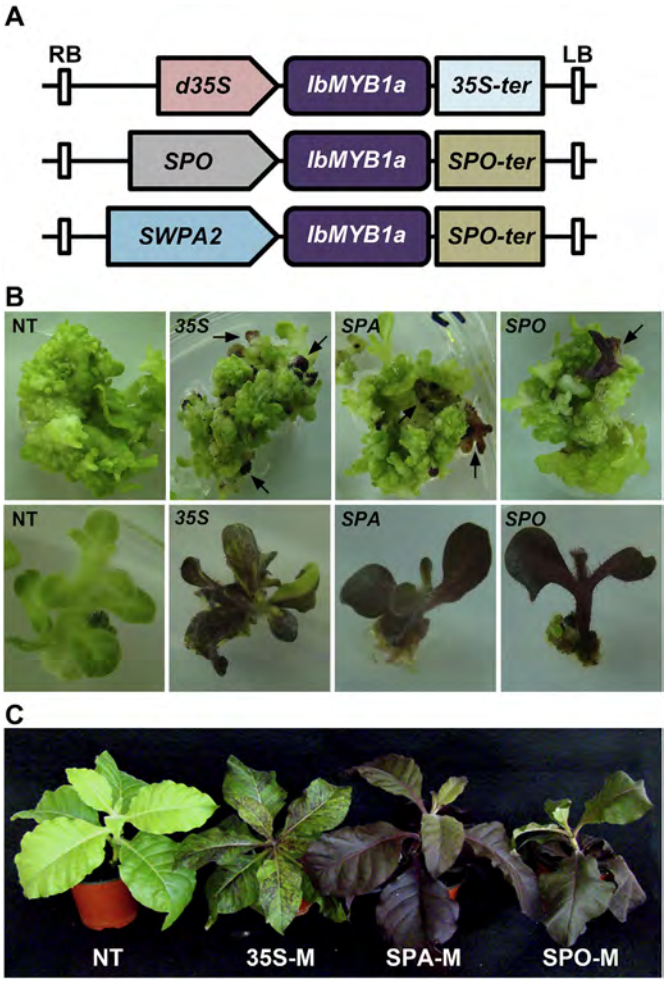

Fig. 1. Generation of transgenic tobacco plants expressing *IbMYB1a*. (A) Schematic representation of the three *IbMYB1a* constructs cloned into binary vector pCambia2300 used for plant transformation. Expression of *IbMYB1a* was driven by the duplicated *CaMV 35S* promoter (*d35S*), the sporamin (*SPO*) promoter, and the peroxidase *SWPA2* (*SPA*) promoter. The *CaMV 35S* terminator was replaced by the sporamin terminator (*SPO-ter*) in both the *SPO* and *SPA* constructs. (B) Regenerating shoots (arrows) from transformed calli showing anthocyanin pigmentation on MS2 regeneration/selection medium containing kanamycin and cefotaxime at 4–6 weeks after transformation (upper panels). Regenerated shoots showing anthocyanin pigmentation on MS basal medium containing kanamycin and cefotaxime at 8–10 weeks after transformation (lower panels). (C) Mature plants (T0) in a growth chamber showing anthocyanin pigmentation. NT, non-transgenic WT tobacco plant; 35S-M, *d35S-IbMYB1a*-OX line; SPA-M, *SWPA2-IbMYB1a*-OX line; SPO-M, *SPO-IbMYB1a*-OX line.

5.4- and 3.4-fold increase over that of the 35S-M and SPA-M lines, respectively. The 35S-M line contained 206 μg anthocyanin/g fresh weight, and the SPA-M line contained 697 μg anthocyanin/g fresh weight. We further analyzed the levels of anthocyanin pigments in the leaves of transgenic tobacco plants using HPLC (Fig. 3B). Complete hydrolysis by boiling crude anthocyanin extract in acidic methanol solvent released the core anthocyanidin (aglycones) component at a retention time of 10.8 min. This retention time corresponded to that of the cyanidin standard (10.894). The HPLC chromatograms indicates that cyanidin was the predominant core molecule of anthocyanidins recovered from the leaves of all three types of *IbMYB1a*-OX lines (Fig. 3B). No anthocyanins were detected in WT leaves. Consistently, the highest levels of anthocyanidins were detected in the SPO-M line, followed by the SPA-M and 35S-M lines. These results suggest that transcriptional expression of *IbMYB1a* in transgenic tobacco plants induced the production of the cyanidin-based anthocyanins as the major pigments.

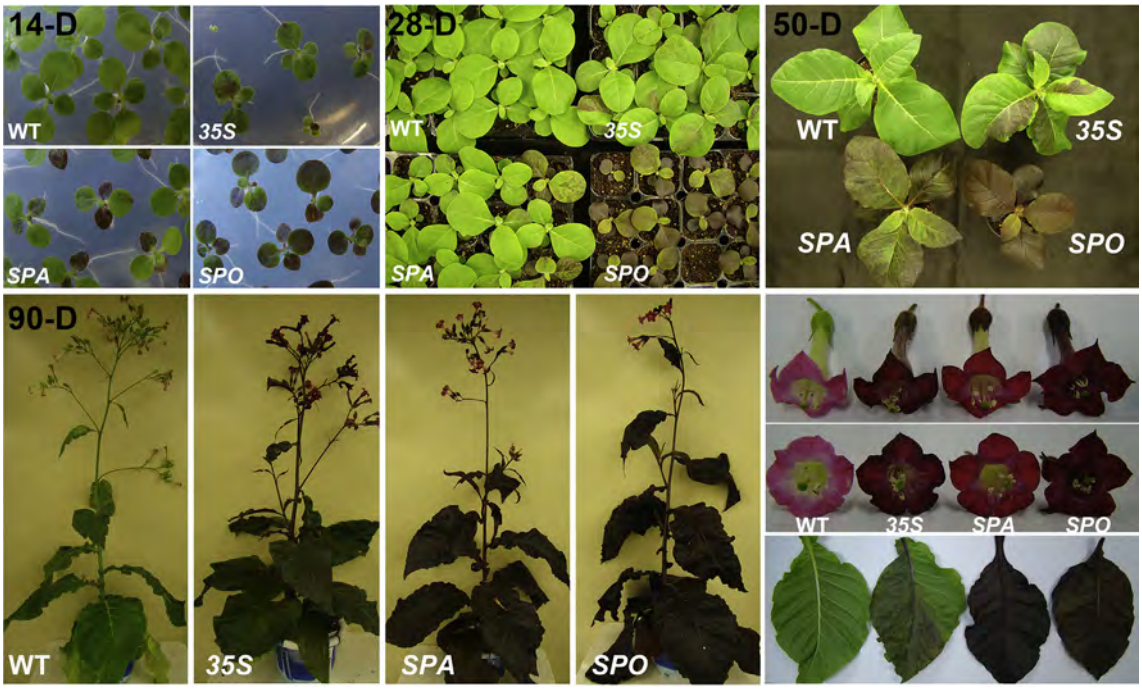

Fig. 2. Comparison of typical anthocyanin phenotypes during growth in three transgenic tobacco plants (T1) expressing *IbMYB1a*. Anthocyanin-accumulating phenotypes in WT and transgenic tobacco plants (35S, SPA, and SPO) were compared at different growth stages (14, 28, 50, and 90 days after germination). Pink flowers from WT plants and red flowers from transgenic plants are shown in the middle panels on the right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Expression of IbMYB1a increases the transcript levels of anthocyanin biosynthetic genes in transgenic tobacco plants

We previously reported that the expression of *IbMYB1a* controls the transcriptional activation of anthocyanin biosynthetic genes and anthocyanin accumulation in *Arabidopsis* plants (Chu et al., 2013). To further elucidate the molecular mechanisms underlying anthocyanin pigment accumulation via expression of *IbMYB1a* in transgenic tobacco plants, we examined the transcript levels of anthocyanin structural genes in both 30-day-old and 60-day-old leaf tissues of each transgenic line (Fig. 4). We did not observe marked differences in the expression levels and patterns of structural genes in between these samples. RT-PCR analysis revealed high levels of *IbMYB1* transcripts in all three *IbMYB1a*-OX lines; these levels were much higher in the SPA-M and SPO-M lines than in the 35S-M line. However, no *IbMYB1a* transcripts were detected in WT plants, which served as a control. These results suggest that the *SPO* and *SWPA2* promoters more strongly control the transcriptional activation of *IbMYB1a* than the *35S* promoter in tobacco plants. Up-regulation of *IbMYB1a* increased the expression of anthocyanin biosynthetic genes encoding PAL, C4H, 4CL, CHS, CHI, F3H, DFR, and ANS. In particular, the expression levels of the late structural genes such as *DFR* and *ANS* in the *IbMYB1a*-OX lines were much higher than those observed in the WT. High expression levels of the structural genes were detected in the SPA-M and SPO-M lines, whereas the transcript levels of these genes in the 35S-M were slightly low. In addition, to see whether up-regulation of *IbMYB1a* affects the gene expression of other regulatory partners such as bHLH and WD40 proteins, we checked the expression patterns of endogenous bHLH and WD40 genes in the *IbMYB1a*-OX transgenic tobacco plants. As shown in Fig. 4, the expression level of *NtAn1* (accession number: HQ589210) encoding bHLH TF increased in all the *IbMYB1a*-OX lines but the transcript levels of *NtMYC1* (accession number: GQ859158) and *NtMYC2* (accession number: GQ859160) genes were not changed. Tobacco genes encoding WD40 repeat protein such as *NtWDR* (accession number: GQ260131) and *NtTTG2* (accession number: FJ795022) were also not affected by *IbMYB1a* expression. This result suggests that IbMYB1a TF may function by inducing the expression of *NtAn1* to regulate anthocyanin biosynthesis. As shown Figs. 3 and 4, the differential expression levels of anthocyanin biosynthetic genes in response to *IbMYB1a* expression correlated well with the anthocyanin accumulation level observed in all three types of *IbMYB1a*-OX lines. These results suggest that *IbMYB1a* expression positively controls the expression of multiple anthocyanin biosynthetic genes at the transcriptional level and induces anthocyanin accumulation in transgenic tobacco plants.

3.5. Anthocyanin accumulation increases the total contents of phenolic compounds and antioxidant activities in transgenic tobacco plants

Since anthocyanins possess high antioxidant properties, we examined the total contents of phenolic compounds, DPPH radical scavenging activity, and antioxidant enzyme activities in leaf samples from each transgenic tobacco plant using spectrophotometric methods (Fig. 5). The levels of total phenolic compounds were higher in transgenic plants than in WT, but these levels significantly varied among the three *IbMYB1a*-OX transgenic tobacco lines. The total phenolic contents were highest in the SPA-M line, followed by the SPO-M and 35S-M lines, although the anthocyanin level was higher in the SPO-M line than in SPA-M (Fig. 5A). DPPH radical scavenging activity was also higher in both the SPA-M and SPO-M lines than in the WT or 35S-M lines but, compared to WT, their activity was relatively low in the transgenic lines (Fig. 5B). The activities of antioxidant enzymes APX and CAT were significantly higher in all three transgenic lines than in WT (Fig. 5C and D). The specific APX enzyme activity in proportion to the total

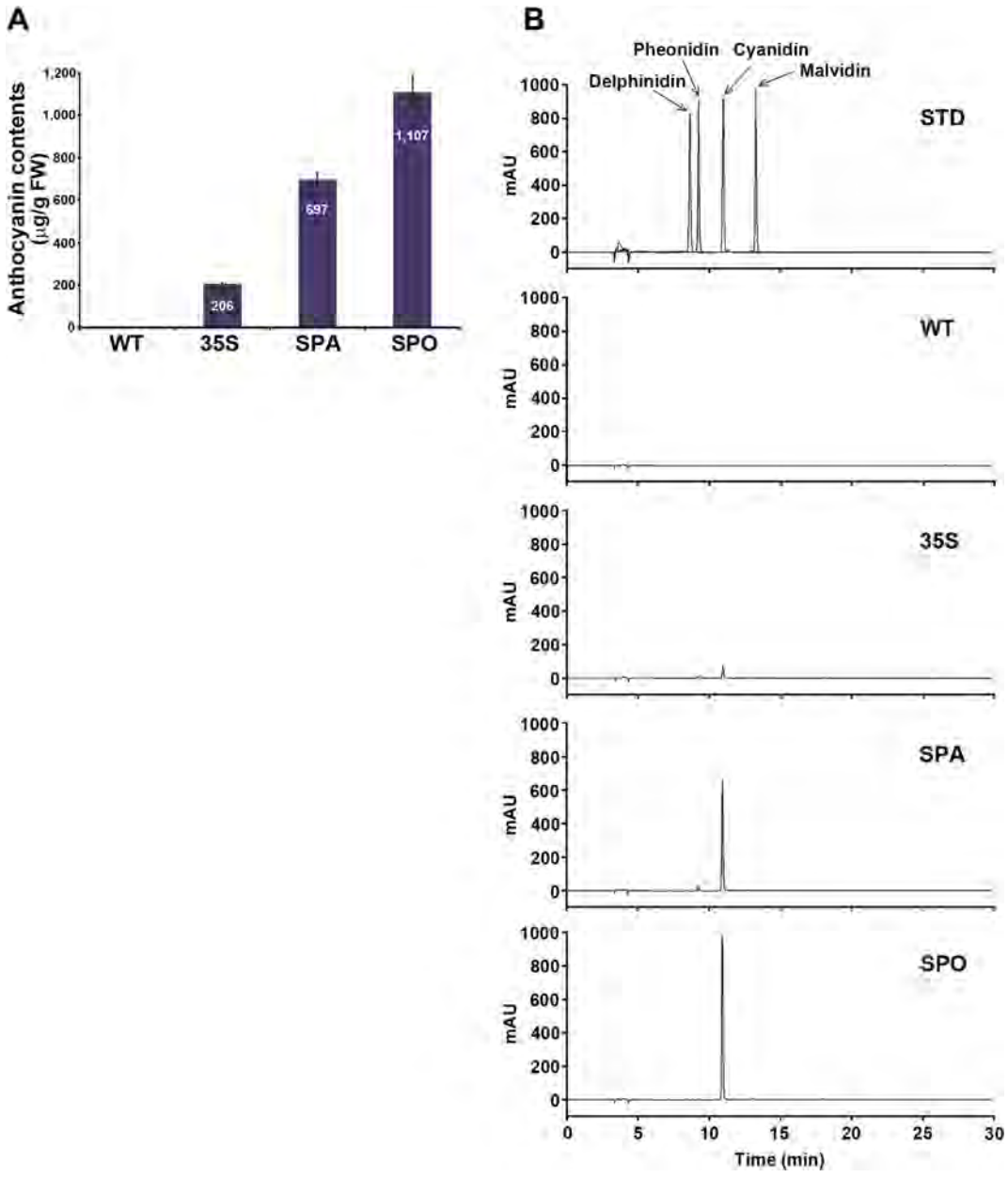

Fig. 3. Anthocyanin quantification and HPLC analysis of the three *IbMYB1a*-OX transgenic tobacco lines. A, Total anthocyanin contents in 50-day-old leaves of tobacco plants. Total anthocyanin contents were spectrophotometrically determined by measuring the absorption of the extracts at wavelengths of 530 nm and 657 nm as described in the Materials and methods. B, HPLC analysis of anthocyanidins derived from acid hydrolysis of the anthocyanins produced in tobacco plants. Elution profiles of tobacco samples and anthocyanidin standards (STD) after reverse phase HPLC are shown. Absorbance was measured at 530 nm. Four anthocyanidin standards (delphinidin, peonidin, cyanidin, and malvidin) were used as controls. WT, wild type; *35S*, *d35S-IbMYB1a*-OX; *SPA*, *SWPA2-IbMYB1a*-OX; *SPO*, *SPO-IbMYB1a*-OX.

phenolic compounds was higher in all *IbMYB1a*-OX transgenic lines than in WT. APX activity in the SPA-M line was considerably higher than that of WT, while slightly higher activity was observed in the SPO-M line than in the 35S-M line. Similarly, specific CAT enzyme activity was highest in the SPA-M line followed by 35S-M and SPO-M. These results suggest that the higher antioxidant activities observed in the transgenic tobacco plants are related to anthocyanin accumulation in the leaves.

4. Discussion

The results show that overexpression of *IbMYB1a*, an alternatively spliced variant of the R2R3-MYB *IbMYB1* gene from sweetpotato, produced anthocyanin pigmentation in all tissues, including leaves, stems, roots, and flowers in tobacco. The use of different promoters driving *IbMYB1a* led to differential expression of this *IbMYB1a* regulatory gene, thereby causing differential expression of several structural genes involved in anthocyanin biosynthesis. These transcriptional alterations led to variations in anthocyanin accumulation in the leaf tissues of transgenic tobacco plants. The phenotypic differences in anthocyanin accumulation and distribution in the leaves of *IbMYB1a*-OX transgenic plants may have been due to differences in promoter activity as well as the availability of endogenous regulatory proteins, such as bHLH and WD40, which interact with the IbMYB1a MYB protein. Such transcriptional regulators in tobacco plants remain to be identified. In

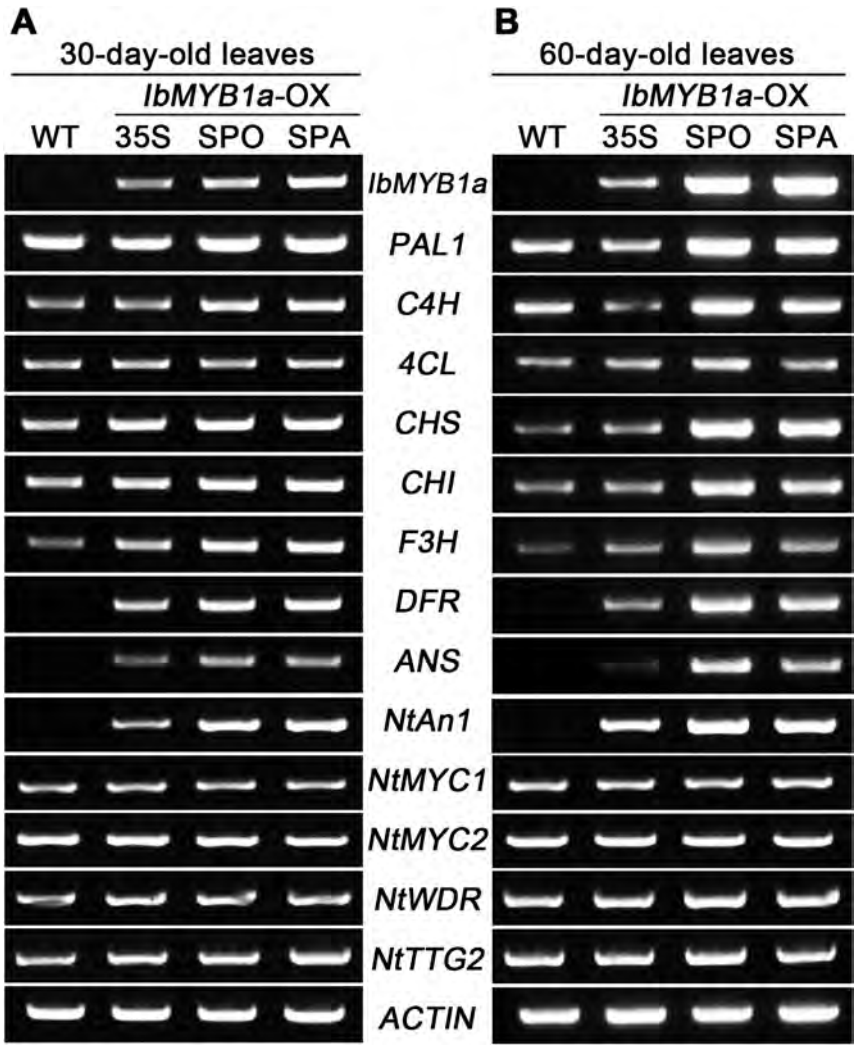

Fig. 4. Expression analysis of anthocyanin biosynthetic genes in the three *IbMYB1a*-OX transgenic tobacco lines by RT-PCR. Expression patterns in 30-day-old (A) and 60-day-old (B) leaves of tobacco plants (T1 lines). Sample tissues from each of the two independent lines were subjected to RT-PCR analysis. Thirty cycles of PCR amplification were performed as described in the Materials and methods. Tobacco *β-ACTIN* was used as a quantitative control.

general, different promoters differentially affect to the expression of transgenes. In the present study, the anthocyanin levels were highest in the SPO-M line and was lowest in the 35S-M line harboring the *d35S* promoter (Fig. 3). This phenotypic difference provides some interesting insights into the activity of these three promoters in transgenic tobacco plants. The *SPO* and *SWPA2* promoters exhibited higher promoter activity than the *d35S* promoter, as exhibited by differences in anthocyanin production in the leaves of tobacco plants. This result indicates that the sweetpotato *SPO* and *SWPA2* promoters are more active than the *d35S* promoter in the leaf tissues of transgenic tobacco.

A powerful expression system with an appropriate promoter is an important prerequisite for efficient expression of desired genes in plant species. To maximize the expression levels of desired genes, it is important to identify suitable promoters that are highly active in target plants. Since the *SPO* promoter was highly active in controlling the expression of the *IbMYB1a* transgene in tobacco, this promoter can be used to express a gene of interest in tobacco at a high level. The *SPO* promoter, however, is not likely a good choice to study and engineer genes whose expression could be detrimental for the host plants, since SPO-M lines with high levels of anthocyanin pigments exhibited negative effects on plant growth. Sporamin is the most abundant protein in storage roots of sweetpotato, accounting for 60–80% of the total soluble protein (Hattori et al., 1990; Yeh et al., 1997). Thus, the sporamin promoter is commonly used for transgene expression in storage roots of sweetpotato. In addition, the use of the sporamin promoter leads to high levels of expression of transgenes in heterologous plants, such as potato, tobacco, *Brassica*, and *Arabidopsis* (Chen et al., 2006; Chu et al., 2013; Hattori et al., 1990; Hong et al., 2008; Ohta et al., 1991; Wang et al., 2002). Moreover, the sporamin gene is systemically expressed in leaves and stems upon wounding (Wang et al., 2002; Yeh et al., 1997). The sporamin promoter is highly responsive to mechanical wounding and methyl jasmonate (MeJA) treatment in the leaves and stems of sweetpotato and transgenic tobacco plants (Wang et al., 2002). These observations, together with the results of the current study, suggest that the *SPO* promoter could be used to drive high levels of transgene expression in heterologous plants. *SWPA2*, encoding an anionic peroxidase (POD), was previously isolated from suspension cultures of sweetpotato. *SWPA2* is predominantly expressed in cultured cells of sweetpotato and is highly induced by environmental stresses, such as wounding, chilling, and ozone in sweetpotato leaves (Kim et al., 1999). Likewise, the *SWPA2* promoter is shown to be strongly induced in transgenic tobacco

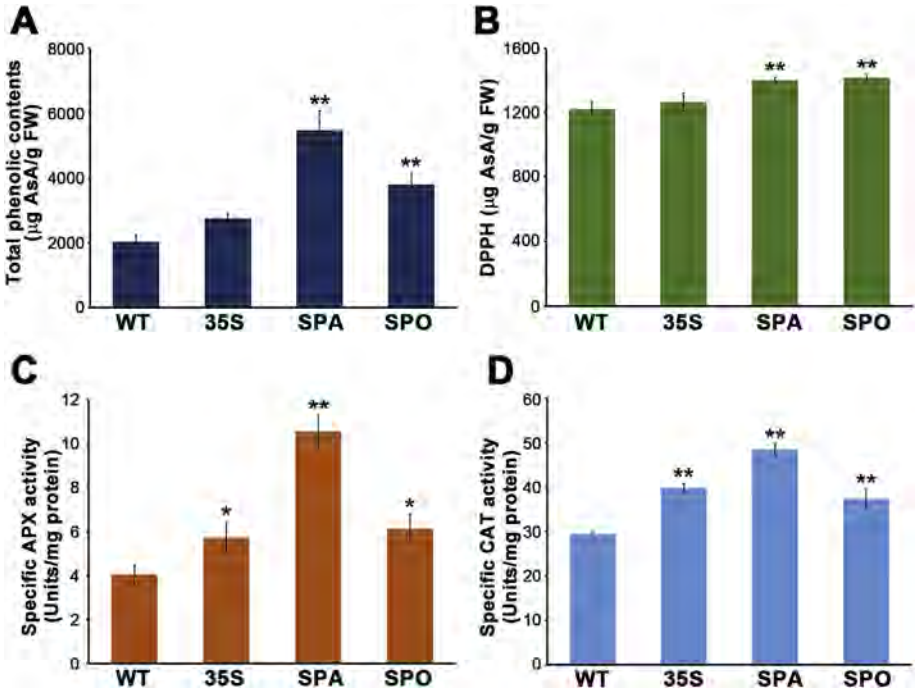

Fig. 5. Effects of anthocyanin accumulation on total phenolic contents (A), DPPH radical scavenging activities (B), and antioxidant enzyme activities of APX (C) and CAT (D) in the three *IbMYB1a*-OX transgenic tobacco lines. Sample tissues were taken from each of the two independent lines for these assays. The results represent mean ± standard error of three independent experiments. All analyses were performed using the SPSS software and statistical significance was set at *P < 0.05 and **P < 0.01.

plants in response to environmental stresses, such as hydrogen peroxide, wounding, and UV treatment (Kim et al., 2003). Moreover, our previous and present studies have shown that the *SWPA2* promoter more strongly induces anthocyanin accumulation and transgene expression than the *d35S* promoter in *Arabidopsis* and tobacco plants (Chu et al., 2013; Figs. 3 and 4). These results suggest that the *SWPA2* promoter could be used to induce high levels of transgene expression in heterologous plants. Based on the analysis of three promoters used for anthocyanin production in tobacco plants, we suggest that *SWPA2* promoter is likely suitable for the use of *IbMYB1a* as a visible marker for plant transformation. Because *SWPA2* promoter did not show any negative effects on plant growth and induced high levels of anthocyanin pigmentation in tobacco plants.

The regulatory TFs R2R3-MYB, bHLH, and WD40 proteins control multiple enzymatic steps in the anthocyanin biosynthetic pathway (Dubos et al., 2010; Ramsay and Glover, 2005; Zimmermann et al., 2004). In general, R2R3-MYB TFs combine with bHLH and WD40 proteins to form a ternary complex (MBW) that activates anthocyanin biosynthetic genes in plants. Among these, co-expression of two classes of TFs, R2R3-MYB and bHLH, is indispensable for the activation of anthocyanin biosynthetic genes. Recently, tobacco bHLH and MYB TFs involved in flavonoid biosynthetic pathway have been reported (Bai et al., 2011; Pattanaik et al., 2010). Tobacco *NtAn2* (accession number: FJ472650) gene encoding an R2R3-MYB TF was isolated from developing tobacco flowers (Pattanaik et al., 2010). Overexpression of *NtAn2* induced whole-plant anthocyanin production in tobacco and *Arabidopsis*. In addition, ectopic expression of *NtAn1* encoding a bHLH TF enhanced anthocyanin accumulation in tobacco flowers. Yeast two-hybrid assays showed that NtAn2 protein interacts with bHLH TF, NtAn1 (Bai et al., 2011). Interestingly, our RT-PCR analysis (Fig. 4) showed that overexpression of *IbMYB1a* up-regulates the expression of *NtAn1* in tobacco. However, the tobacco R2R3-MYB *NtAn2*, which was known to interact with NtAn1, did not responded to *IbMYB1a* expression (data not shown). This finding indicates that IbMYB1a protein can combine with endogenous tobacco NtAn1 TF by inducing its expression to activate anthocyanin biosynthetic genes such as DFR and ANS in tobacco plants. It is also possible that certain bHLH and WD40 TFs with its constitutive expression can participate in forming a ternary complex of MYB-bHLH-WD40.

To date, many R2R3-MYB regulatory genes from various plant species have been shown to activate the expression of structural metabolic enzymes that induce anthocyanin biosynthesis. Their availability for anthocyanin production has been examined via heterologous expression analysis of the *MYB* gene in transgenic *Arabidopsis* and tobacco plants. R2R3-MYB proteins such as ZmC1, PAP1, MdMYB10, and VvMYBA1 require a bHLH partner for anthocyanin biosynthesis (Espley et al., 2007; Goff et al., 1992; Hichri et al., 2011; Walker et al., 2007; Zimmermann et al., 2004). Interestingly, overexpression of *PAP1* increases transcript levels of the *TT8* (bHLH) gene in *Arabidopsis* (Tohge et al., 2005). We have previously and consistently demonstrated that heterologous expression of *IbMYB1a* induces the expression of endogenous *PAP1* and *TT8* genes in transgenic *Arabidopsis* (Chu et al., 2013). In addition, when *PAP1* alone was infiltrated into the leaves of *Nicotiana benthamiana*, no anthocyanin pigmentation was observed (data not shown). By contrast, transfection of *IbMYB1a* in tobacco using a transient agro-infiltration assays led to the accumulation of anthocyanin in tobacco leaves (Kim et al., 2010). These reports suggest that PAP1 MYB protein requires the presence of TT8 bHLH protein for anthocyanin biosynthesis in *Arabidopsis*. However, our previous and present studies have shown that IbMYB1a does not require other regulatory factors such as bHLH for anthocyanin biosynthesis in transgenic *Arabidopsis* or tobacco plants (Chu et al., 2013; Fig. 2). Likewise, overexpression of grape *VlMYBA2* produces anthocyanin red pigmentation in several dicot plants, such as tobacco and

Arabidopsis (Geekiyanage et al., 2007). Recently, Huang et al. (2013) also showed that overexpression of R2R3-MYB *EsMYBA1* alone induces strong anthocyanin accumulation in transgenic tobacco and *Arabidopsis* via up-regulation of the main flavonoid-related genes. These results indicate that the expression of a single R2R3-MYB regulatory gene alone is sufficient for the activation of anthocyanin biosynthesis without the need for co-expression of another regulatory partner bHLH protein. This finding highlights a significant advantage of metabolic engineering of anthocyanins in various plant species, since MYB TFs such as IbMYB1a, VlMYBA2, and EsMYBA1 TFs do not depend on their bHLH partner for anthocyanin pigmentation in dicot plants, including *Arabidopsis* and tobacco. Indeed, we also found that ectopic expression of *IbMYB1a* induced purple pigmentation in alfalfa in addition to *Arabidopsis* and tobacco plants (data not shown).

Our previous and present studies suggest that expression of *IbMYB1a* in tobacco and *Arabidopsis* seems to be promising for the identification of the transformants. As described previously for *IbMYB1a*-OX transgenic *Arabidopsis* plants, all primary tobacco transformants and their progenies displayed anthocyanin pigmentation. This distinguishable phenotype is an indication that *IbMYB1a* can be used for visual identification of transformed tissues in *Arabidopsis* and tobacco plants. This finding suggests that the *IbMYB1a* gene can potentially be used as a simple, non-destructive visible marker for plant transformation. Several *MYB* genes have already been identified as possible candidates for the use in an anthocyanin visible marker system, which would serve as a safe, efficient selectable marker system for plant transformation (Geekiyanage et al., 2007; Goldsbrough et al., 1996; Kim et al., 2010; Li et al., 2011; Ludwig et al., 1990). The utilization of *MYB* genes not only enables simple, versatile selection of transformed tissues through visualization, but it may also increase the nutritional value of a transgenic plant by increasing its anthocyanin levels. However, the potential use of these genes as visible markers should be further investigated throughout the transgenic plant production process, from the efficient selection and recovery of transformed regenerants during transformation to the monitoring of transgenic plants.

Stresses that stimulate anthocyanin production were shown to increase the activities of antioxidant enzymes such as CAT, APX, and superoxide dismutase (SOD) in leaves (Agati et al., 2012; Kumchai et al., 2013; Neill et al., 2002). The enzyme activities of APX and CAT increased in proportion to the anthocyanin content, as observed in all the *IbMYB1a*-OX lines (Figs. 3 and 5). However, the antioxidant enzyme activities and total phenolic content were less in the SPO-M than in the SPA-M lines, despite the highest level of anthocyanins in the SPO-M lines. This suggests that excess content of anthocyanins may decrease the activities of antioxidant enzymes, although appropriate anthocyanin accumulation increases the enzyme activities of CAT and APX. Furthermore, the SPO-M lines caused plant growth retardation due to high levels of anthocyanin pigments. Thus, it is possible that high accumulation of anthocyanins may negatively affect the antioxidant enzyme activities such as CAT and APX. However, more studies are needed to find out a positive or negative correlation between anthocyanin content and antioxidant enzyme activities.

Author contributions

C.Y. Kim and C.H. An: conceived and designed the experiments. C.H. An, K.-W. Lee, S.-H. Lee, Y.J. Jeong and S.G. Woo: performed the experiments. H. Jeon, Y.-I. Park and S.S. Kwak: analyzed the data. K.-W. Lee, S.-H. Lee, S.S. Kwak: contributed reagents/materials/analysis tools. C.Y. Kim and C.H. An: wrote the paper.

Acknowledgments

This work was supported by grants from the KRIBB Initiative Program and the Next-Generation BioGreen 21 Program (SSAC, grant #: PJ01107004), Rural Development Administration, Republic of Korea.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.02.002.

References

Aebi, H., 1984. [13] Catalase *in vitro*. In: Lester, P. (Ed.), Methods in Enzymology. Academic Press, pp. 121–126.
Agati, G., Azzarello, E., Pollastri, S., Tattini, M., 2012. Flavonoids as antioxidants in plants: location and functional significance. Plant Sci. 196, 67–76.
Ainsworth, E.A., Gillespie, K.M., 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat. Protoc. 2, 875–877.
Allan, A.C., Hellens, R.P., Laing, W.A., 2008. MYB transcription factors that colour our fruit. Trends Plant Sci. 13, 99–102.
Bai, Y., Pattanaik, S., Patra, B., Werkman, J., Xie, C., Yuan, L., 2011. Flavonoid-related basic helix-loop-helix regulators, NtAn1a and NtAn1b, of tobacco have originated from two ancestors and are functionally active. Planta 234, 363–375.
Ban, Y., Honda, C., Hatsuyama, Y., Igarashi, M., Bessho, H., Moriguchi, T., 2007. Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. Plant Cell Physiol. 48, 958–970.
Bartel, B., Matsuda, S.P., 2003. Plant biology. Seeing red. Science 299, 352–353.
Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A., Lamb, C., 2000. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell 12, 2383–2394.
Chen, H.J., Wang, S.J., Chen, C.C., Yeh, K.W., 2006. New gene construction strategy in T-DNA vector to enhance expression level of sweet potato sporamin and insect resistance in transgenic *Brassica oleracea*. Plant Sci. 171, 367–374.
Chu, H., Jeong, J.C., Kim, W.J., Chung, D.M., Jeon, H.K., Ahn, Y.O., Kim, S.H., Lee, H.S., Kwak, S.S., Kim, C.Y., 2013. Expression of the sweetpotato R2R3-type *IbMYB1a* gene induces anthocyanin accumulation in *Arabidopsis*. Physiol. Plant 148, 189–199.
Cutanda-Perez, M.C., Ageorges, A., Gomez, C., Vialet, S., Terrier, N., Romieu, C., Torregrosa, L., 2009. Ectopic expression of *VlmybA1* in grapevine activates a narrow set of genes involved in anthocyanin synthesis and transport. Plant Mol. Biol. 69, 633–648.
Deluc, L., Bogs, J., Walker, A.R., Ferrier, T., Decendit, A., Merillon, J.M., Robinson, S.P., Barrieu, F., 2008. The transcription factor *VvMYB5b* contributes to the regulation of anthocyanin and proanthocyanidin biosynthesis in developing grape berries. Plant Physiol. 147, 2041–2053.
Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., Lepiniec, L., 2010. MYB transcription factors in *Arabidopsis*. Trends Plant Sci. 15, 573–581.
Espley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E., Kutty-Amma, S., Allan, A.C., 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor, *MdMYB10*. Plant J. 49, 414–427.
Geekiyanage, S., Takase, T., Ogura, Y., Kiyosue, T., 2007. Anthocyanin production by over-expression of grape transcription factor gene *VlmybA2* in transgenic tobacco and *Arabidopsis*. Plant Biotechnol. Rep. 1, 11–18.
Goff, S.A., Cone, K.C., Chandler, V.L., 1992. Functional analysis of the transcriptional activator encoded by the maize B gene: evidence for a direct functional interaction between two classes of regulatory proteins. Genes Dev. 6, 864–875.
Goldsbrough, A.P., Tong, Y., Yoder, J.I., 1996. *Lc* as a non-destructive visual reporter and transposition excision marker gene for tomato. Plant J. 9, 927–933.
Hattori, T., Nakagawa, S., Nakamura, K., 1990. High-level expression of tuberous root storage protein genes of sweet potato in stems of plantlets grown *in vitro* on sucrose medium. Plant Mol. Biol. 14, 595–604.
Hichri, I., Barrieu, F., Bogs, J., Kappel, C., Delrot, S., Lauvergeat, V., 2011. Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J. Exp. Bot. 62, 2465–2483.
Holton, T.A., Cornish, E.C., 1995. Genetics and biochemistry of Anthocyanin biosynthesis. Plant Cell. 7, 1071–1083.
Hong, Y.F., Liu, C.Y., Cheng, K.J., Hour, A.L., Chan, M.T., Tseng, T.H., Chen, K.Y., Shaw, J.F., Yu, S.M., 2008. The sweet potato sporamin promoter confers high-level phytase expression and improves organic phosphorus acquisition and tuber yield of transgenic potato. Plant Mol. Biol. 67, 347–361.
Horsch, R.B., Klee, H.J., 1986. Rapid assay of foreign gene expression in leaf discs transformed by *Agrobacterium tumefaciens*: role of T-DNA borders in the transfer process. Proc. Natl. Acad. Sci. U. S. A. 83, 4428–4432.
Huang, W., Sun, W., Lv, H., Luo, M., Zeng, S., Pattanaik, S., Yuan, L., Wang, Y., 2013. A R2R3-MYB transcription factor from *Epimedium sagittatum* regulates the flavonoid biosynthetic pathway. PLoS One 8, e70778.

Kim, C.Y., Ahn, Y.O., Kim, S.H., Kim, Y.H., Lee, H.S., Catanach, A.S., Jacobs, J.M., Conner, A.J., Kwak, S.S., 2010. The sweet potato *IbMYB1* gene as a potential visible marker for sweet potato intragenic vector system. Physiol. Plant 139, 229–240.

Kim, K.Y., Huh, G.H., Lee, H.S., Kwon, S.Y., Hur, Y., Kwak, S.S., 1999. Molecular characterization of cDNAs for two anionic peroxidases from suspension cultures of sweet potato. Mol. Gen. Genet. 261, 941–947.

Kim, K.Y., Kwon, S.Y., Lee, H.S., Hur, Y., Bang, J.W., Kwak, S.S., 2003. A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. Plant Mol. Biol. 51, 831–838.

Kobayashi, S., Ishimaru, M., Hiraoka, K., Honda, C., 2002. *Myb*-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. Planta 215, 924–933.

Kumchai, J., Huang, J.Z., Lee, C.Y., Chen, F.C., Chin, S.W., 2013. The induction of antioxidant enzyme activities in Cabbage seedlings by heavy metal stress. Eng. Technol. 12, 5589–5601.

Li, Z.T., Dhekney, S.A., Gray, D.J., 2011. Use of the *VvMybA1* gene for non-destructive quantification of promoter activity via color histogram analysis in grapevine (*Vitis vinifera*) and tobacco. Transgenic Res. 20, 1087–1097.

Ludwig, S.R., Bowen, B., Beach, L., Wessler, S.R., 1990. A regulatory gene as a novel visible marker for maize transformation. Science 247, 449–450.

Mano, H., Ogasawara, F., Sato, K., Higo, H., Minobe, Y., 2007. Isolation of a regulatory gene of anthocyanin biosynthesis in tuberous roots of purple-fleshed sweet potato. Plant Physiol. 143, 1252–1268.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 473–497.

Nagata, T., Todoriki, S., Masumizu, T., Suda, I., Furuta, S., Du, Z., Kikuchi, S., 2003. Levels of active oxygen species are controlled by ascorbic acid and anthocyanin in *Arabidopsis*. J. Agric. Food Chem. 51, 2992–2999.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880.

Neill, S.O., Gould, K.S., Kilmartin, P.A., Mitchell, K.A., Markham, K.R., 2002. Antioxidant activities of red versus green leaves in *Elatostema rugosum*. Plant Cell Environ. 25, 539–547.

Ohta, S., Hattori, T., Morikami, A., Nakamura, K., 1991. High-level expression of a sweet potato sporamin gene promoter: beta-glucuronidase (GUS) fusion gene in the stems of transgenic tobacco plants is conferred by multiple cell type-specific regulatory elements. Mol. Gen. Genet. 225, 369–378.

Park, S.C., Kim, Y.H., Kim, S.H., Jeong, Y.J., Kim, C.Y., Lee, J.S., Bae, J.Y., Ahn, M.J., Jeong, J.C., Lee, H.S., Kwak, S.S., 2015. Overexpression of the IbMYB1 gene in an orange-fleshed sweetpotato cultivar produces a dual-pigmented transgenic sweetpotato with improved antioxidant activity. Physiol. Plant 86, 82–90.

Pattanaik, S., Kong, Q., Zaitlin, D., Werkman, J., Xie, C., Patra, B., Yuan, L., 2010. Isolation and functional characterization of a floral tissue-specific R2R3 MYB regulator from tobacco. Planta 231, 1061–1076.

Petroni, K., Tonelli, C., 2011. Recent advances on the regulation of anthocyanin synthesis in reproductive organs. Plant Sci. 181, 219–229.

Ramsay, N.A., Glover, B.J., 2005. MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. Trends Plant Sci. 10, 1360–1385.

Rodriguez-Bonilla, L., Cuevas, H.E., Montero-Rojas, M., Bird-Pico, F., Luciano-Rosario, D., Siritunga, D., 2014. Assessment of genetic diversity of sweet potato in puerto rico. PLoS One 9, e116184.

Sharma, O.P., Bhat, T.K., 2009. DPPH antioxidant assay revisited. Food Chem. 113, 1202–1205.

Stitt, M., Sulpice, R., Keurentjes, J., 2010. Metabolic networks: how to identify key components in the regulation of metabolism and growth. Plant Physiol. 152, 428–444.

Tanaka, Y., Sasaki, N., Ohmiya, A., 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. Plant J. 54, 733–749.

Teow, C.C., Truong, V.D., McFeeters, R.F., Thompson, R.L., Pecota, K.V., Yencho, G.C., 2007. Antioxidant activities, phenolic and β-carotene contents of sweet potato genotypes with varying flesh colours. Food Chem. 103, 829–838.

Tohge, T., Nishiyama, Y., Hirai, M.Y., Yano, M., Nakajima, J., Awazuhara, M., Inoue, E., Takahashi, H., Goodenowe, D.B., Kitayama, M., Noji, M., Yamazaki, M., Saito, K., 2005. Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. Plant J. 42, 218–235.

Vimolmangkang, S., Han, Y., Wei, G., Korban, S.S., 2013. An apple MYB transcription factor, MdMYB3, is involved in regulation of anthocyanin biosynthesis and flower development. BMC Plant Biol. 13, 176.

Walker, A.R., Lee, E., Bogs, J., McDavid, D.A., Thomas, M.R., Robinson, S.P., 2007. White grapes arose through the mutation of two similar and adjacent regulatory genes. Plant J. 49, 772–785.

Wang, S.J., Lan, Y.C., Chen, S.F., Chen, Y.M., Yeh, K.W., 2002. Wound-response regulation of the sweet potato sporamin gene promoter region. Plant Mol. Biol. 48, 223–231.

Winkel-Shirley, B., 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126, 485–493.

Yeh, K.W., Chen, J.C., Lin, M.I., Chen, Y.M., Lin, C.Y., 1997. Functional activity of sporamin from sweet potato (*Ipomoea batatas* Lam.): a tuber storage protein with trypsin inhibitory activity. Plant Mol. Biol. 33, 565–570.

Zimmermann, I.M., Heim, M.A., Weisshaar, B., Uhrig, J.F., 2004. Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like bHLH proteins. Plant J. 40, 22–34.