

## Expression of the sweetpotato R2R3-type *IbMYB1a* gene induces anthocyanin accumulation in *Arabidopsis*

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R2R3-type MYB transcription factors (TFs) play important roles in transcriptional regulation of anthocyanins. The R2R3-type *IbMYB1* is known to be a key regulator of anthocyanin biosynthesis in the storage roots of sweetpotato. We previously showed that transient expression of *IbMYB1a* led to anthocyanin pigmentation in tobacco leaves. In this article, we generated transgenic *Arabidopsis* plants expressing the *IbMYB1a* gene under the control of *CaMV 35S* promoter, and the sweetpotato *SPO* and *SWPA2* promoters. Overexpression of *IbMYB1a* in transgenic *Arabidopsis* produced strong anthocyanin pigmentation in seedlings and generated a deep purple color in leaves, stems and seeds. Reverse transcription-polymerase chain reaction analysis showed that *IbMYB1a* expression induced upregulation of several structural genes in the anthocyanin biosynthetic pathway, including *4CL*, *CHI*, *F3'H*, *DFR*, *AGT*, *AAT* and *GST*. Furthermore, overexpression of *IbMYB1a* led to enhanced expression of the *AtTT8* (*bHLH*) and *PAP1/AtMYB75* genes. High-performance liquid chromatography analysis revealed that *IbMYB1a* expression led to the production of cyanidin as a major core molecule of anthocyanidins in *Arabidopsis*, as occurs in the purple leaves of sweetpotato (cv. Sinzami). This result shows that the *IbMYB1a* TF is sufficient to induce anthocyanin accumulation in seedlings, leaves, stems and seeds of *Arabidopsis* plants.

### Introduction

Anthocyanins are water-soluble secondary metabolites belonging to the class of flavonoids that confer the purple, red and blue hues present in flowers, fruits,

vegetables, grains and storage organs (Harborne and Grayer 1988, Tanaka et al. 2008). They are synthesized in the cytosol from phenylalanine and stored in vacuoles. Anthocyanins, with a basic structure of C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub>, are highly modified compounds that are normally

**Abbreviations** – 4CL, 4-coumarate-CoA ligase; AAT, anthocyanin acyltransferase; AGT, anthocyanidin glycosyltransferase; AN2, *Anthocyanin2*; ANS, anthocyanidin synthase; bHLH, basic helix-loop-helix; C1, *Colorless1*; C4H, cinnamate 4-hydroxylase; CaMV, cauliflower mosaic virus; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3'H, flavonoid 3'-hydroxylase; F3H, flavanone-3-hydroxylase; GST, glutathione S-transferase; HPLC, high-performance liquid chromatography; PAL, phenylalanine ammonia-lyase; PAP1, *PRODUCTION OF ANTHOCYANIN PIGMENT1*; PI, *Purple plant PI*; R, *Red*; RT-PCR, reverse transcription-polymerase chain reaction; SPO, sporamin; SWPA2, sweetpotato anionic peroxidase2; TFs, transcription factors; TT8, *TRANSPARENT TESTA8*.

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attached to one or more glycoside groups. A total of 19 types of anthocyanidins (aglycone chromophores of anthocyanins) are known, but six of these are common, such as cyanidin, pelargonidin, delphinidin, peonidin, petunidin and malvidin. The structures of these chromophores can contain numerous glycosidic substitutions at the C3 and C5 positions (Tanaka et al. 2008). Additional variations often occur by acylation of the sugar groups with organic acids. The most widespread anthocyanins found in leaves are the glycosides of cyanidin (Ribereau-Gayon 1972).

MYB transcription factors (TFs) play important roles in transcriptional regulation of diverse secondary metabolites collectively known as flavonoids (Paz-Ares et al. 1987, Martin and Paz-Ares 1997, Jin and Martin 1999), Quattrocchio et al. 1999, Nesi et al. 2001). *MYB* genes are widely distributed in higher plants and comprise one of the largest families of TFs. In the *Arabidopsis* genome, there are 198 genes of the *MYB* superfamily, consisting of 126 *R2R3-MYB*, 5 *R1R2R3-MYB*, 64 *MYB*-related and 3 atypical *MYB* genes (Yanhui et al. 2006, Dubos et al. 2010). Most *R2R3-MYB* TFs were reported to play vital roles in the regulation of secondary metabolism in the flavonoid biosynthetic pathway (Jin and Martin 1999, Borevitz et al. 2000, Stracke et al. 2001). The first identified plant *R2R3-MYB* gene was *Colorless1 (C1)* from maize, which regulates structural genes that encode enzymes of the anthocyanin biosynthetic pathway (Paz-Ares et al. 1987). Similar to *C1*, some *R2R3-MYB* family members, such as petunia *AN2* (Quattrocchio et al. 1999), *Arabidopsis PAP1/AtMYB75* and *PAP2/AtMYB90* (Borevitz et al. 2000), maize *Purple plant (Pl)* (Cone et al. 1993), grape *MYBA* (Kobayashi et al. 2002), and apple *MdMYB10* (Espley et al. 2007), were shown to control anthocyanin biosynthesis in plants. For example, overexpression of *PAP1* in transgenic *Arabidopsis* led to upregulation of a number of genes in the anthocyanin biosynthesis pathway from phenylalanine ammonia-lyase (PAL) to chalcone synthase (CHS) and dihydroflavonol 4-reductase (DFR) (Borevitz et al. 2000, Tohge et al. 2005). The apple *MdMYB10* gene was shown to control anthocyanin biosynthesis in apple fruit in a red-fleshed cultivar and in the skin of other varieties (Espley et al. 2007). Recently, Mano et al. (2007) reported that the *R2R3*-type *IbMYB1* from the purple-fleshed sweetpotato (cv. Ayamurasaki) was responsible for purple pigmentation in the flesh of storage roots. To date, a number of regulatory genes involved in anthocyanin production have been isolated from several plant species. The transcriptional regulators for anthocyanin biosynthesis include three components of an *R2R3-MYB* protein, a bHLH (basic

helix-loop-helix, MYC) protein and WD40-type co-regulators (WD40). It was shown that combinations of the *R2R3-MYB*, bHLH and WD40 proteins and their interactions determine the subset of genes that are expressed in plant species (Mol et al. 1998, Springob et al. 2003, Broun 2005, Koes et al. 2005, Ramsay and Glover 2005). The maize *R2R3-MYB* factor *ZmC1*, together with its bHLH partner *ZmR* (Red), stimulates the accumulation of cyanidin derivatives which are the major pigments found in maize tissues (Grotewold et al. 1998).

Anthocyanins are important antioxidant molecules, thus providing nutritional value for humans and help to protect plants from oxidative damage (Nagata et al. 2003). Thus, understanding the regulatory function of the *R2R3-MYB* gene in anthocyanin biosynthesis is essential for the purpose of metabolic engineering of anthocyanins in food crops. Furthermore, there is increasing interest in development of alternative selection strategies to avoid the use of bacterial antibiotics and herbicides in plant transformation (Kim et al. 2010, Kortstee et al. 2011). The *R2R3-MYB* gene responsible for anthocyanin production has potential as a visible selectable marker gene because of its coloration during plant transformation. Kortstee et al. (2011) reported that expression of the apple *MdMYB10* in apple and strawberry resulted in increased anthocyanin accumulation in explants during transformation. The sweetpotato *IbMYB1* gene was also evaluated as an intragenic color-selectable marker in sweetpotato transformation (Kim et al. 2010). Transient expression of *IbMYB1a* in tobacco led to anthocyanin accumulation in infiltrated leaves by activating structural genes involved in the anthocyanin biosynthetic pathway.

In this report, we tested the ability of the sweetpotato *IbMYB1a* to activate the anthocyanin biosynthetic pathway in *Arabidopsis* plants. We generated transgenic *Arabidopsis* lines that overexpress *IbMYB1a* under the control of three different promoters, including constitutive *35S* promoter, the sweetpotato root-specific sporamin (*SPO*) promoter, and the sweetpotato oxidative stress-inducible peroxidase (*SWPA2*) promoter. Heterologous expression of the *IbMYB1a* in *Arabidopsis* led to the stimulation of a subset of genes involved in the anthocyanin biosynthetic pathway and induced high levels of anthocyanin production. Our results demonstrate that *IbMYB1a* TF controls anthocyanin accumulation in *Arabidopsis* through transcriptional regulation of several structural genes involved in anthocyanin biosynthesis. These results suggest that the single *IbMYB1a* TF can be exploited for the production of anthocyanins, which have important nutritive values in plant species of interest.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used in this study. Seeds were surface-sterilized in 70% ethanol for 3 min, and then three times for 1 min each in 95% ethanol. Seeds were dried on filter paper and transferred to half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 1% sucrose and 0.8% phyto agar (Duchefa). Plates were placed at 4°C for 2 days for stratification and then placed under long-day growth conditions (16 h light/8 h dark cycle) at 22°C for 10–16 days. Healthy seedlings were transplanted to soil on trays and covered with a plastic lid. Four-week-old, fully expanded healthy leaves were used for all experiments, except where noted otherwise.

### Agrobacterium-mediated transformation into *Arabidopsis*

The sweetpotato *IbMYB1a* cDNA was cloned into the pCambia2300 binary vector as described by Kim et al. (2010). The *IbMYB1a* constructs in the pCambia2300 binary vectors with different promoters were transformed into *Agrobacterium tumefaciens* strain GV3101. Stable transgenic *Arabidopsis* plants were generated using the floral dip method (Clough and Bent 1998). Transformants were selected on half-strength MS medium supplemented with kanamycin (50 µg ml<sup>-1</sup>) and well-rooted seedlings were transplanted to soil for experimental analysis and seed set.

### RNA extraction and reverse transcriptase-polymerase chain reaction analysis

Total RNA was extracted from the frozen samples (approximately 0.2 g) using an easy-spin™ IIp plant RNA extraction kit (iNtRON, Korea) according to the manufacturer's instructions. The concentration of RNA was quantified at 260 nm by spectrophotometric measurement. First strand cDNAs were synthesized from 2.5 µg of total RNA using a RevertAid™ first-strand cDNA synthesis kit (Fermentas, Waltham, MA). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed in 50 µl reactions containing 2 µl of 1:10 diluted cDNA samples and gene-specific primers using Ex-Taq DNA polymerase (TaKaRa, Japan) under the following conditions: an initial denaturation step at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, polymerization at 72°C for 1 min, and a final extension at 72°C for 10 min. Control RT-PCR was performed using a

primer pair specific to the *ACTIN2* (At3g18780) gene under the same conditions. From each reaction, 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 Appendix S1, Supporting Information. Primers were designed using primer3 software (Rozen and Skaletsky 2000).

### Extraction of anthocyanins and absorbance measurements

Anthocyanins were extracted from 0.2 g of finely ground plant material as described by Mehrtens et al. (2005) with minor modifications. Samples were extracted with 1 ml of acidic methanol containing 1% HCl (v/v) for 18 h at room temperature under moderate shaking. Plant materials were sedimented by centrifugation at 16 800 g for 15 min, and 500 µl of the supernatant was mixed with 500 µl of MilliQ H<sub>2</sub>O and 300 µl of chloroform in a 2.0-ml microcentrifuge tube to remove chlorophyll in the extraction. After centrifugation at 8600 g for 5 min, the supernatant (water–methanol phase) was transferred to a new tube. Absorption of the extracts at 530 and 657 nm wavelength was determined spectrophotometrically with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Quantification of anthocyanins was performed using the following equation:  $Q_{\text{Anthocyanins}} = (A_{530} - 0.25 \times A_{657}) \times M^{-1}$ , where  $Q_{\text{Anthocyanins}}$  is the amount of anthocyanins,  $A_{530}$  and  $A_{657}$  are the absorptions at the indicated wavelengths, and  $M$  is the weight of the plant material used for extraction (g). All samples were measured as triplicates in two independent biological replicates. Error bars indicate the standard deviation (SD) of the average of the anthocyanin content.

### Hydrolysis of anthocyanins

Anthocyanins were hydrolyzed by boiling as described by Zhou et al. (2008). One hundred microliters of the methanol extraction as described above were added to 900 µl of solvent [95:5 (v/v), *n*-butanol (100%):HCl (36%)] in a 1.5-ml microcentrifuge tube. The mixture was boiled for 1 h to release core anthocyanidins. After the sample was cooled to room temperature, it was dried using a speed vacuum at room temperature. The residue was dissolved in 100 µl of 0.1% HCl–methanol (0.1% HCl in HPLC-grade methanol) solvent and centrifuged at 14 500 g for 10 min. The supernatant was used to identify core anthocyanidin molecules using high performance liquid chromatography (HPLC) analysis.

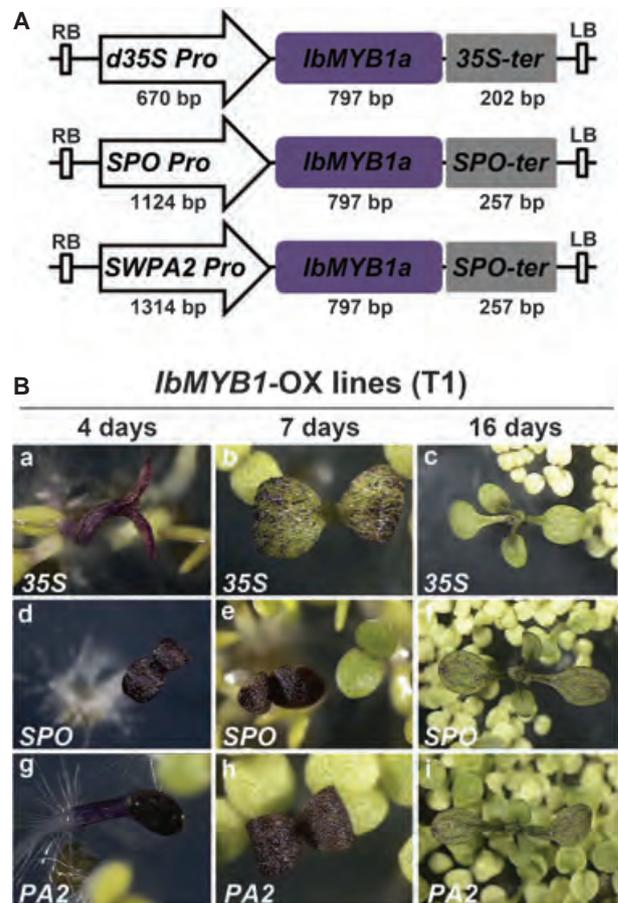
## HPLC analysis of anthocyanidin profile

The analysis of anthocyanin profiles in *Arabidopsis* samples was carried out using HPLC. The HPLC analysis was performed with an Agilent series 1200 quaternary solvent delivery system, cooled autosampler (4°C), and photodiode array detector (Agilent Technologies, Santa Clara, CA). The samples were separated on a 4.5 × 250 mm, 5 μm YMC ODS column. The column was maintained at 30°C. The volume of injection was 10 μl. The mobile phase consisted of (A) 1% phosphoric acid and 10% acetic acid in water, and (B) acetonitrile with the following gradient: 5–40% B (0–5 min), 5–40% B (5–25 min), and 40% B (25–30 min), with a flow rate of 1 ml min<sup>-1</sup>. All chromatograms were monitored at 520 nm. The standards cyanidin, delphinidin, pelargonidin and peonidin (Sigma-Aldrich, St. Louis, MO) were used for identification and quantification.

## Results

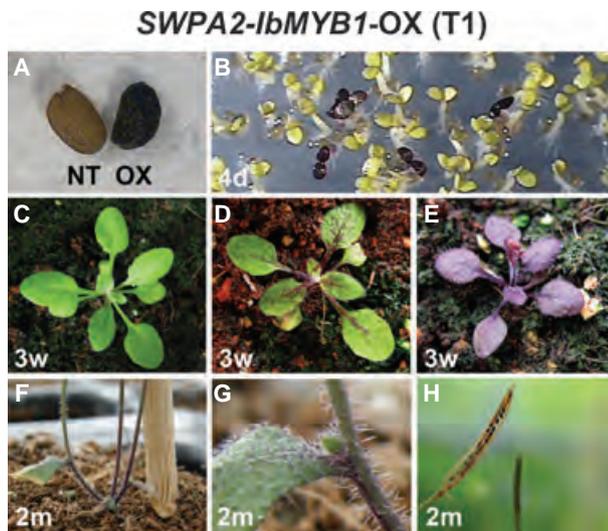
### Overexpression of *IbMYB1a* in *Arabidopsis* causes anthocyanin pigmentation in various tissues and organs

We previously reported that the *IbMYB1* gene produces two splice variants of *IbMYB1a* and *IbMYB1b* by alternative splicing. We observed that transient expression of the *IbMYB1a* splice variant in tobacco leaves induced anthocyanin accumulation (Kim et al. 2010). To further examine the sweetpotato *IbMYB1a* as a potential regulator of anthocyanin biosynthesis in *Arabidopsis*, transgenic *Arabidopsis* plants expressing the *IbMYB1a* gene were generated under the control of three different promoters, including the duplicated 35S promoter, the sweetpotato sporamin *SPO* promoter, and the sweetpotato peroxidase *SWPA2* promoter (Fig. 1A). As expected, we were able to identify primary transformants as black seeds after harvesting from floral-dipped plants that were transformed with the three *IbMYB1a*-expression constructs, because of the strongly pigmented embryos visible through the seed coat. Through continuous screening of transformants on half-strength MS agar plates containing kanamycin, we obtained multiple transgenic *Arabidopsis* lines (T1) with a striking purple phenotype from each construct. All three types of *IbMYB1a*-overexpression (OX) transgenic *Arabidopsis* lines were readily distinguished from wild type by their purple color (Fig. 1B). Expression of *IbMYB1a* in transgenic *Arabidopsis* plants displayed striking purple coloration because of anthocyanin accumulation in their cotyledons and hypocotyls. Thus, it appears that the sweetpotato promoters *SPO* and *SWPA2* successfully drove high expression of *IbMYB1a*



**Fig. 1.** Overexpression of *IbMYBa* in *Arabidopsis* plants (T1). (A) Schematic representation of the three *IbMYB1a* constructs in the binary vector pCambia2300 for plant transformation. Expression of *IbMYB1a* was driven by the control of duplicated *CaMV* 35S promoter, sweetpotato sporamin *SPO* promoter or sweetpotato peroxidase *SWPA2* (*PA2*) promoter. The *CaMV* 35S terminator was replaced by the sporamin terminator (*SPO-ter*) in the sweetpotato promoter-*IbMYB1a* expression cassettes. Sizes of promoter, terminator and cDNA are shown in base pairs (bp). (B) Transgenic phenotypes of *IbMYB1a* overexpression in seedlings (4–7 days old) and young plants (16 days old). Anthocyanin accumulation in hypocotyls and cotyledons (a, b, d, e, g, h) and in young leaves (c, f, i). Images were taken at the indicated times with a digital camera (AxioCam MRC, Carl Zeiss).

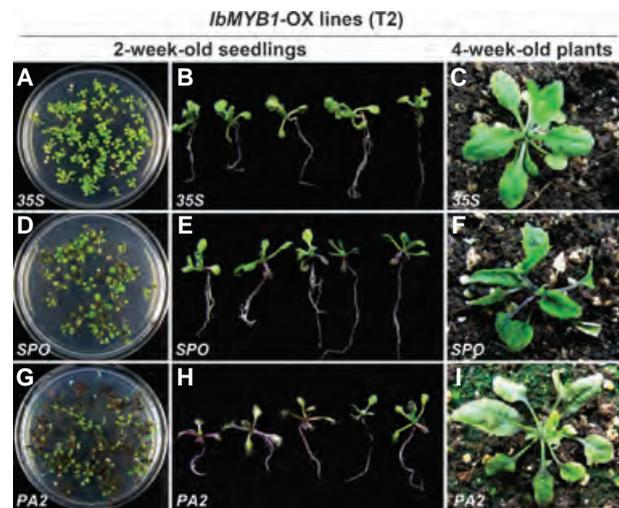
in heterologous *Arabidopsis* plants. During germination of each *IbMYB1a*-OX line (T1), the seedlings from all three constructs exhibited strong anthocyanin pigmentation in cotyledons and hypocotyls except in roots and root hairs. However, as the seedlings developed over 16 days, pigmentation became reduced in cotyledons and hypocotyls. Anthocyanins also accumulated in shoot tips and juvenile leaves (Fig. 1B: c, f, i). It appeared that seedlings with strong anthocyanin pigmentation produced slightly longer cotyledons with a wrinkled surface (Fig. 1B: f, i). The *IbMYB1a*-OX lines from the three



**Fig. 2.** Anthocyanin phenotypes of transgenic *Arabidopsis* plants (T1) with *SWPA2-IbMYB1a* constructs during development. (A) Wild-type (NT) and *SWPA2-IbMYB1a* transgenic (OX) seeds. (B) 4-day-old seedlings. (C) 3-week-old wild-type plant. (D, E) 3-week-old *SWPA2-IbMYB1a* plants. (F, G) 2-month-old *SWPA2-IbMYB1a* stems and trichomes. (H) 2-month-old *SWPA2-IbMYB1a* siliques.

constructs were phenotypically similar but varied in the extent of anthocyanin accumulation. The highest pigmentation in seedlings was observed in *SWPA2-IbMYB1a*-OX lines. *35S-IbMYB1a*-OX lines exhibited a somewhat low anthocyanin accumulation in *Arabidopsis* tissues compared with the other two transgenic lines. Thus, we further observed anthocyanin phenotypes at different developmental stages with *SWPA2-IbMYB1a*-OX lines (Fig. 2). Multiple transgenic lines with various purple pigments were produced and they could be readily distinguished from wild type, showing that anthocyanin pigments were detectable in seeds, seedlings, rosette leaves, stems and trichomes. Furthermore, transgenic seeds in siliques for *SWPA2-IbMYB1a*-OX lines were visibly black (Fig. 2H). Some transgenic lines with massive anthocyanin accumulation, as shown in Fig. 2E, did not reach maturity and eventually died before setting seeds. Likewise, anthocyanin pigments were also detectable in trichomes and flowers of the three *IbMYB1a*-OX lines (see Appendix S2).

We also examined anthocyanin phenotypes in the T2 generation of the *IbMYB1a*-OX lines (Fig. 3). Overall, transgenic T2 plants displayed purplish plants that are characteristic of anthocyanin accumulation, similar to the transgenic T1 plants, but T2 plants exhibited reduced anthocyanin levels compared with T1 plants. Much less anthocyanin accumulated in seedlings and rosette leaves from *35S-IbMYB1a*-OX lines (Fig. 3A–C). High levels of anthocyanins were

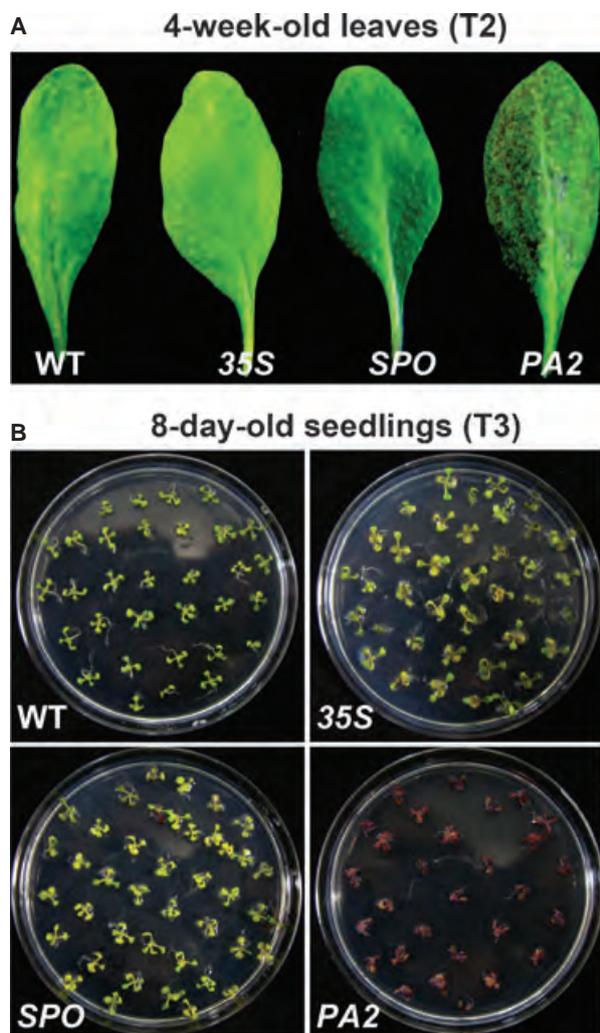


**Fig. 3.** Anthocyanin phenotypes of transgenic *Arabidopsis* plants (T2) with *d35S-IbMYB1a* (*35S*), *SPO-IbMYB1a* (*SPO*) and *SWPA2-IbMYB1a* (*PA2*) constructs. (A, B, D, E, G, H) 2-week-old seedlings grown on half-strength MS medium plate. (C, F, I) 4-week-old transgenic plants grown in soil.

observed in seedlings and rosette leaves from the T2 generation of *SWPA2-IbMYB1a*-OX lines (Fig. 3G–I), similar to the anthocyanin levels in T1 plants. Transgenic seedlings with strong anthocyanin pigments, especially in *SWPA2-IbMYB1a*-OX, did not grow normally to maturity. Thus, we selected transgenic lines displaying moderate anthocyanin accumulation without aberrant growth. High anthocyanin pigmentation was observed in cotyledons, petioles, leaf veins and roots of *SPO-IbMYB1a*-OX and *SWPA2-IbMYB1a*-OX lines. Taken together, anthocyanin pigmentation in *IbMYB1a*-OX *Arabidopsis* plants was similar to that previously observed in *pap1-D* activation tagging mutants and *35S-PAP1* overexpressing plants (Borevitz et al. 2000). This indicates that *IbMYB1a* is the sweetpotato ortholog of the *PAP1* gene, which is a well-known anthocyanin regulator in *Arabidopsis*. These results suggest that expression of *IbMYB1a* in heterologous *Arabidopsis* plants is sufficient for anthocyanin production in seeds, seedlings, rosette leaves, stems and trichomes.

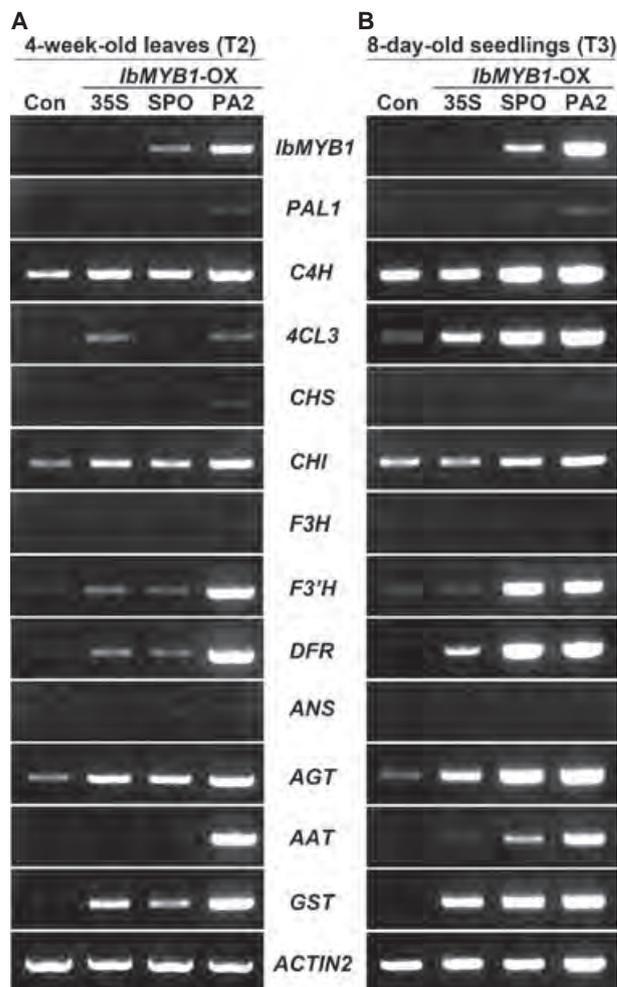
### Overexpression of *IbMYB1a* elevates transcript levels of structural genes in anthocyanin biosynthetic pathways

We examined anthocyanin accumulation in 4-week-old rosette leaves (T2) and 8-day-old seedlings (T3) of *IbMYB1a*-OX lines. As shown in Fig. 4, anthocyanin pigments were greatly reduced in rosette leaves



**Fig. 4.** Anthocyanin accumulation in transgenic *Arabidopsis* plants with *d35S-IbMYB1a* (35S), *SPO-IbMYB1a* (SPO) and *SWPA2-IbMYB1a* (PA2) constructs. (A) Anthocyanin phenotypes in 4-week-old rosette leaves (T2). (B) Anthocyanin phenotypes in 8-day-old seedlings (T3). A representative line from the two independent lines was taken for photography.

from the three constructs. Almost undetectable anthocyanin pigments were observed in *35S-IbMYB1a*-OX lines and very low levels of anthocyanins accumulated in *SPO-IbMYB1a*-OX lines. By contrast, purplish leaves with anthocyanin pigments developed in *SWPA2-IbMYB1a*-OX lines (Fig. 4A). Weak anthocyanin pigments were detected in T3 seedlings of *35S-IbMYB1a*-OX and *SPO-IbMYB1a*-OX lines, whereas strong anthocyanin pigments were observed in T3 seedlings of *SWPA2-IbMYB1a*-OX lines (Fig. 4B). We performed standard RT-PCR experiments to verify that overexpression of *IbMYB1a* regulated transcript levels of structural genes involved in anthocyanin biosynthetic



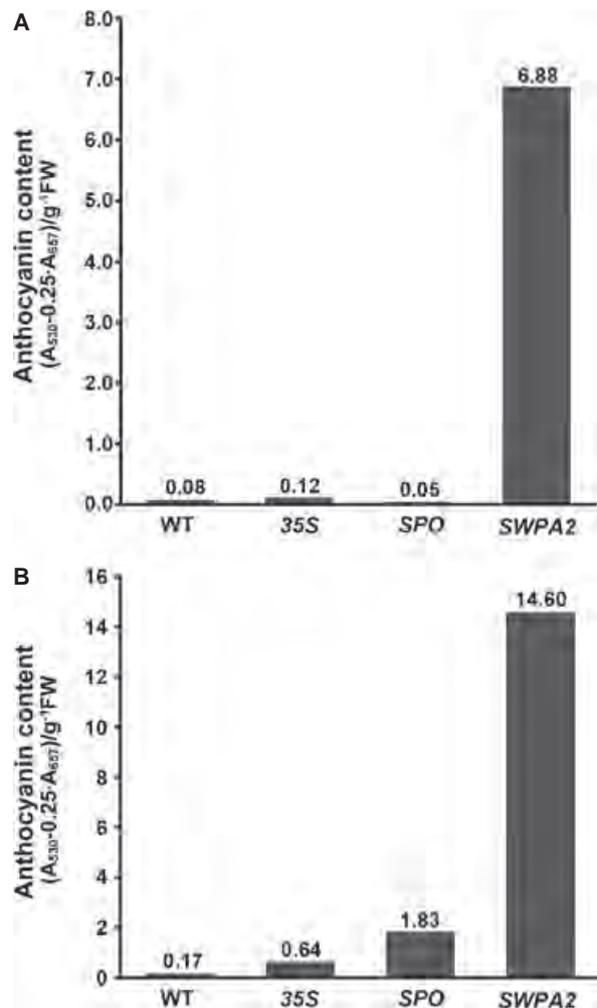
**Fig. 5.** Expression analysis of anthocyanin biosynthetic genes by RT-PCR in the three *IbMYB1a*-OX transgenic *Arabidopsis* plants. (A) Expression patterns in 4-week-old rosette leaves (T2) lines. (B) Expression patterns in 8-day-old seedlings (T3) lines. Con, wild type; 35S, *d35S-IbMYB1a*-OX; SPO, *SPO-IbMYB1a*-OX; PA2, *SWPA2-IbMYB1a*-OX. Sample tissues from each of the two independent lines were collected for RT-PCR analysis. Thirty cycles of PCR amplification were performed as described in section Materials and methods. *ACTIN2* was used as a quantitative control. *Arabidopsis* anthocyanin biosynthetic genes used for RT-PCR are as follows: *PAL1*, phenylalanine ammonia-lyase 1; *C4H*, cinnamate-4-hydroxylase; *4CL3*, 4-coumarate-CoA ligase 3; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavanone-3-hydroxylase; *F3'H*, flavonoid 3'-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *ANS*, anthocyanidin synthase; *AGT*, anthocyanin glycosyltransferase; *AAT*, anthocyanin acyltransferase; *GST*, glutathione S-transferase.

pathways in seedlings and rosette leaves. Anthocyanin accumulation has been reported to positively correlate with the expression of anthocyanin biosynthetic genes (Paz-Ares et al. 1987, Borevitz et al. 2000, Espley et al. 2007). Fig. 5 shows that each *IbMYB1a*-OX line produced an increase in the corresponding

*IbMYB1a* transcript in rosette leaf tissues from 4-week-old plants (T2) and 8-day-old seedlings (T3). The expression levels of *IbMYB1a* transcripts were highest in *SWPA2-IbMYB1a-OX* lines, and transcript levels were very low in *35S-IbMYB1a-OX* lines. However, no *IbMYB1a* transcripts were detected in wild-type plants used as a control. In parallel with *IbMYB1a* overexpression, increased expression of anthocyanin biosynthetic genes such as *PAL1*, *cinnamate 4-hydroxylase (C4H)*, *4-coumarate-CoA ligase3 (4CL3)*, *CHS*, *chalcone isomerase (CHI)*, *flavonoid 3'-hydroxylase (F3'H)*, *DFR*, *anthocyanidin glycosyltransferase (AGT)*, *anthocyanin acyltransferase (AAT)* and *glutathione S-transferase (GST)* were observed. The structural genes such as *flavanone-3-hydroxylase (F3H)* and *anthocyanidin synthase (ANS)* were slightly induced by high expression of *IbMYB1a* in 4-week-old rosette leaves (T2), but not in 8-day-old seedlings (T3). In addition, Fig. 5 shows that *35S-IbMYB1a-OX* lines resulted in weak expression of *IbMYB1a* and structural genes compared with the other two constructs. This correlates well with the low levels of anthocyanin accumulation in *35S-IbMYB1a-OX* transgenic plants, suggesting that low levels of anthocyanin accumulation in *35S-IbMYB1a-OX* transgenic plants are due to the weak expression of *IbMYB1a* under the control of the 35S promoter. By contrast, it is likely that high expression of *IbMYB1a* under the control of the sweetpotato *SWPA2* promoter caused strong anthocyanin accumulation in transgenic *Arabidopsis* plants. These results suggest that expression of *IbMYB1a* induces anthocyanin production in *Arabidopsis* plants by elevating the transcript levels of multiple anthocyanin biosynthetic genes.

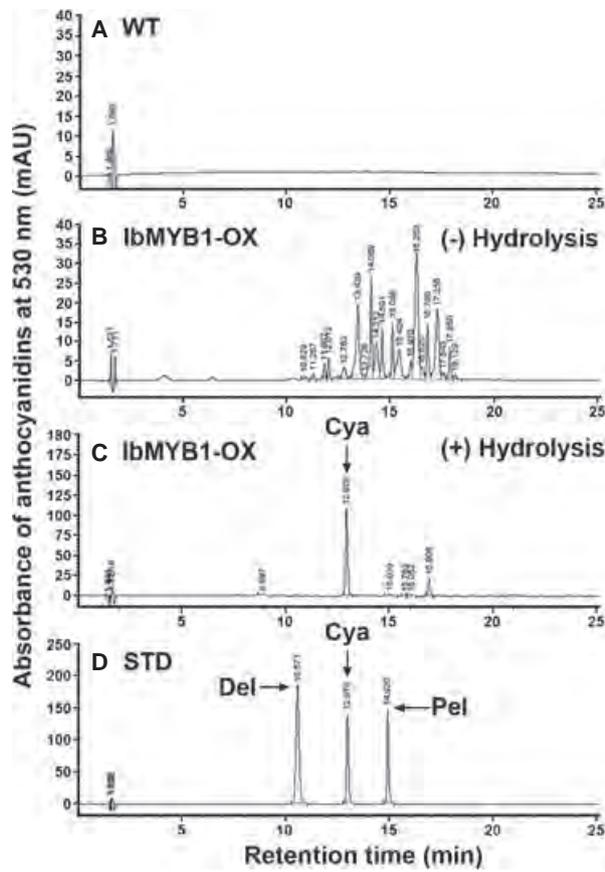
### Overexpression of *IbMYB1a* in transgenic *Arabidopsis* leads to production of cyanidin as a major core molecule of anthocyanidins

We previously reported that transient expression of *IbMYB1a* in tobacco leaves resulted in the accumulation of cyanidin as a core molecule of the anthocyanidins. In addition, we found that cyanidin derivatives are the major anthocyanin pigments formed in the leaves of purple-fleshed sweetpotato cv. Sinzami (see Appendix S3). To confirm whether purple pigmentation observed in all three types of *IbMYB1a-OX* lines is attributed to anthocyanin accumulation, we first measured total anthocyanin content in 4-week-old rosette leaf tissues (T2) and 8-day-old seedlings (T3) of transgenic *Arabidopsis* plants (Fig. 6). For this experiment, we used the same set of tissue samples as shown in Fig. 4. Each tissue sample was extracted with acidic methanol and total anthocyanin content was determined



**Fig. 6.** Anthocyanin quantification in the three *IbMYB1a-OX* transgenic *Arabidopsis* plants. (A) Total anthocyanin content in 4-week-old rosette leaves (T2 lines). (B) Total anthocyanin content in 8-day-old seedlings (T3 lines). WT, wild type; 35S, *d35S-IbMYB1a-OX*; SPO, *SPO-IbMYB1a-OX*; PA2, *SWPA2-IbMYB1a-OX*. Sample tissues from each of the two independent lines were collected for anthocyanin quantification by spectrophotometry. Total anthocyanin content was determined spectrophotometrically by reading absorption of the extracts at wavelengths of 530 nm and 657 nm as described in section Materials and methods.

by spectrometric measurements. Almost 90-fold higher anthocyanin levels were detected in the leaf (Fig. 6A) and seedling (Fig. 6B) tissues from *SWPA2-IbMYB1a-OX* lines compared with wild-type plants. The rosette leaf tissues of *35S-IbMYB1a-OX* and *SPO-IbMYB1a-OX* lines showed similar anthocyanin levels as wild-type (Fig. 6A), whereas seedlings had approximately 4–11-fold higher anthocyanin levels compared with wild type (Fig. 6B). High levels of anthocyanins in *SWPA2-IbMYB1a-OX* lines are correlated with anthocyanin pigments and the observed expression levels of anthocyanin biosynthetic



**Fig. 7.** HPLC analysis of anthocyanidins derived from the acid hydrolysis of anthocyanins produced in *SWPA2-IbMYB1a-OX Arabidopsis* plants. HPLC chromatograms show total anthocyanins (–hydrolysis) and anthocyanidins (+hydrolysis) hydrolyzed from anthocyanins in *IbMYB1a-OX* 8-day-old seedlings (T3). Elution profiles of wild type (WT) without acid hydrolysis and anthocyanidin standards (STD) are shown with reverse phase HPLC. Absorbance was measured at 530 nm. Three standards of anthocyanidins (Cys, cyanidin; Del, delphinidin; Pel, pelargonidin) were used as controls.

genes shown in Figs 4 and 5, respectively. We further analyzed the anthocyanin pigments in the seedling tissues by HPLC (Fig. 7). No anthocyanins were detected in wild-type seedlings, but a variety of anthocyanins were observed in seedlings of *SWPA2-IbMYB1a-OX* lines (Fig. 7A, B). Complete hydrolysis by boiling crude anthocyanin extraction in acidic methanol solvent released the core anthocyanidin component at 12.9 min. HPLC chromatograms showed that cyanidin was the predominant core molecule of the anthocyanidins recovered from the seedlings of *SWPA2-IbMYB1a-OX* lines (Fig. 7C, D). As reported previously in transient expression of *IbMYB1a* in tobacco leaves, this result suggests that overexpression of *IbMYB1a* in transgenic *Arabidopsis* induced the main anthocyanins formed by cyanidin derivatives.

## Discussion

Our results show that heterologous expression of the sweetpotato R2R3-MYB *IbMYB1a* confers transcriptional activation of several structural genes in the anthocyanin biosynthetic pathway, and induces anthocyanin pigmentation in transgenic *Arabidopsis* plants. In our previous report, we tested the potential of the *IbMYB1a* gene as a color-selectable marker for the intragenic vector system in sweetpotato. Transient expression of *IbMYB1a* TF under the control of the sweetpotato promoters *SPO* and *SWPA2* led to upregulation of a set of anthocyanin biosynthetic genes such as *CHS*, *CHI*, *F3H*, *DFR* and *ANS*, and induced the accumulation of anthocyanin pigment in tobacco leaves (Kim et al. 2010). In this article, we investigated the function of the *IbMYB1a* gene in anthocyanin biosynthesis in *Arabidopsis*. The results, together with the previous study, suggest that the *IbMYB1a* TF alone is sufficient to activate anthocyanin biosynthesis through transcriptional regulation in dicyledonous plants such as *Arabidopsis* and tobacco. It may be interesting to check whether expression of *IbMYB1a* alone can lead to anthocyanin production in monocots such as rice and maize.

Coordinated transcriptional regulation of structural biosynthetic genes is a major mechanism that determines the final accumulation of secondary metabolites in plant cells. This regulation is achieved by specific TFs that interact with promoter regions of target genes and thereby modulate the rate of initiation of mRNA synthesis by RNA polymerase II (Ranish and Hahn 1996). Recent studies on the anthocyanin biosynthesis pathway have identified many structural and regulatory genes involved in anthocyanin biosynthesis (Nesi et al. 2001, Winkel-Shirley 2001, Vom Endt et al. 2002, Dubos et al. 2010). Two major classes of TFs, R2R3-MYB and bHLH (MYC), together with a WD40 type co-regulator have been reported to control transcription of the structural genes in the anthocyanin biosynthetic pathway (Cone et al. 1986, Paz-Ares et al. 1987, Ludwig et al. 1989, Ramsay and Glover 2005, Schwinn et al. 2006). The cooperation between R2R3-MYB and bHLH TFs was first demonstrated in maize with the interaction of C1 (MYB) and R (bHLH) for the activation of anthocyanin biosynthesis. The transcriptional activation of anthocyanin biosynthetic genes by the R2R3-MYB proteins ZmC1 or ZmPI1 in *Zea mays* was shown to require a member of the R/B gene family, bHLH. A direct interaction between the MYB domain of ZmC1 and the N-terminal domain of the bHLH protein ZmB has been described (Goff et al. 1992). In addition, the PAP1/AtMYB75 and PAP2/AtMYB90, which regulate structural genes including *PAL*, *CHS*, *DFR* and *GST*

required for anthocyanin production in *Arabidopsis*, were shown to depend on a bHLH partner (Borevitz et al. 2000, Zimmermann et al. 2004). Zimmermann et al. (2004) reported that R2R3-MYB proteins including PAP1/AtMYB75 and PAP2/AtMYB90 have a conserved amino acid signature ([DE]LX<sub>2</sub>[RK]<sub>x3</sub>LX<sub>6</sub>LX<sub>3</sub>R) in the MYB R3 consensus as the structural basis for interaction between MYB and bHLH proteins. These results have demonstrated that anthocyanin production is strictly dependent on the specific bHLH factor expressed in a particular tissue or any plant species. However, our observations in *Arabidopsis* and tobacco plants showed that IbMYB1a TF alone is sufficient to activate anthocyanin biosynthesis through transcriptional regulation of anthocyanin biosynthetic genes. However, we do not exclude the possibility that there is a higher endogenous level of the bHLH protein in the transgenic *Arabidopsis* and tobacco plants. It is also possible that overexpression of *IbMYB1a* can lead to induction of bHLH gene in both plants. Overexpressing lines of PAP1/AtMYB75 have elevated transcript levels of the *AtTT8* gene (At4g09820) that encodes a bHLH protein involved in regulating condensed tannin and anthocyanin biosynthesis (Tohge et al. 2005). Our RT-PCR result confirmed that overexpression of *IbMYB1a* in *Arabidopsis* plants induced enhanced expression of the *AtTT8* gene. We also found that the transcript level of the *PAP1/AtMYB75* gene increased in *IbMYB1a*-OX lines (see Appendix S4). As a result, we demonstrate that high expression of PAP1/AtMYB75 and AtTT8 (bHLH) TFs in *IbMYB1a*-OX lines led to anthocyanin accumulation through transcriptional activation of several structural genes involved in anthocyanin biosynthesis. The amino acid signature that specifies interaction with bHLH proteins was conserved in both MdMYB10 and IbMYB1a TFs. In a study that transiently expressed MdMYB10 in tobacco leaves, it was shown that MdMYB10 required the co-expression of the bHLH protein MdbHLH3 (Espley et al. 2007). By contrast, our previous study revealed that transient expression of a single IbMYB1a TF without exogenous co-expression of bHLH led to high anthocyanin production in tobacco leaves. It is possible that IbMYB1a and MdMYB10 TFs might require other bHLH partners to modulate the expression of structural genes for anthocyanin biosynthesis in tobacco leaves. The data indicate that IbMYB1a is more effective than MdMYB10 in regulating anthocyanin biosynthesis in tobacco leaves. As a further study, isolation of bHLH partners and WD40 co-regulators of IbMYB1a will be very important to better understand the transcriptional regulation of anthocyanin biosynthetic genes by the IbMYB1a-bHLH-WD40 complex.

Previous studies indicate that the expression of anthocyanin biosynthetic genes is coordinately regulated in

response to various developmental and environmental cues (Martin and Paz-Ares 1997, Quattrocchio et al. 1999, Dubos et al. 2010). In *Arabidopsis*, the co-regulated structural genes have been grouped into two subsets: the early biosynthetic genes (EBGs) including *CHS*, *CHI*, *F3H* and *F3'H*, and the late biosynthesis genes (LBGs) such as *DFR*, *ANS* and *UFGT* (*AGT*). The expression of EBGs and LBGs appears to be controlled separately by different TFs (Nesi et al. 2001, Koes et al. 2005, Dubos et al. 2010). For increased anthocyanin production, both EBGs and LBGs need to be coordinately regulated. Our RT-PCR result suggested that IbMYB1a TF seems to control the expression of both the EBGs (*PAL1*, *C4H*, *4CL3*, *CHS*, *CHI* and *F3'H*) and the LBGs (*DFR*, *AGT*, *AAT* and *GST*) at the transcriptional level in *Arabidopsis*. Our previous report also showed that IbMYB1a induced transcript increases of both the EBGs (*CHS*, *CHI* and *F3H*) and the LBGs (*DFR* and *ANS*) in tobacco leaves (Kim et al. 2010). The final step of anthocyanin biosynthesis is the transport of the pigment molecule to the vacuole where the pigments are stabilized and express the full pigment color. The expression of the *GST* gene involved in the vacuolar transport process (Grotewold 2004) was highly induced by overexpression of *IbMYB1a* in seedlings and leaves of transgenic *Arabidopsis*. This indicates that the IbMYB1a TF may control the vacuolar transport of anthocyanin pigments through transcriptional regulation of the *GST* gene. Our DMACA (dimethylaminocinnamaldehyde) staining to analyze the accumulation of proanthocyanidins indicated that no accumulation of proanthocyanidins was detectable in the both seedlings and leaves of transgenic *Arabidopsis* (data not shown). The result shows that the IbMYB1a TF does not regulate proanthocyanidin biosynthesis.

Because of the health-promoting properties of anthocyanins, there is currently a growing interest in the development of functional food and feed crops rich in anthocyanins. The identification of key regulatory genes controlling anthocyanin pigmentation in plants makes it possible to improve the existing commercial varieties and to render them as functional foods and feeds. Instead of modifying single enzymatic steps, it has been suggested that coordinate control of multiple steps with the help of key regulatory genes would lead to an even more predictable control of metabolic flux (Broun 2004, Capell and Christou 2004). In this respect, the single IbMYB1a TF offers promise for metabolic engineering of anthocyanin content in food and feed crops, because it has the potential to activate multiple enzymatic steps in anthocyanin biosynthesis. Indeed, we have shown that the IbMYB1a TF is able to control multiple steps in anthocyanin biosynthetic pathways

in tobacco and *Arabidopsis* plants. These data suggest that *IbMYB1a* TF would be very attractive to develop transgenic plants rich in anthocyanin pigments in plant species of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Primer sequences used for RT-PCR in this study.

**Appendix S2.** Anthocyanin accumulation in trichomes and flowers of the three *IbMYB1a-OX Arabidopsis* plants (T1).

**Appendix S3.** HPLC analysis of anthocyanidins derived from the acid hydrolysis of anthocyanins produced in leaves of sweetpotato cultivars.

**Appendix S4.** Expression analysis of anthocyanin regulatory genes in *Arabidopsis* by RT-PCR in the three *IbMYB1a-OX Arabidopsis* plants.