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Genetically engineered anthocyanin pathway for high health-promoting pigment production in eggplant

Yanjie Zhang · Guihua Chu · Zongli Hu · Qiong Gao · Baolu Cui · Shibing Tian · Bo Wang · Guoping Chen

Abstract Anthocyanins, natural pigments with high antioxidant activities, are widely distributed in the plant kingdom and play important roles in various physiological processes. Much effort has been committed to enhancing the anthocyanin content of these health-promoting pigments in vegetables and grains, for their eye-catching colors and special nutrients. Previously, we reported that the *SmMYB1* gene encoding a R2R3 MYB transcription factor participated in the regulation of anthocyanin biosynthesis in the peel of eggplant. In this work, we introduced *SmMYB1* into a non-anthocyanin-accumulating eggplant cultivar (*Solanum aethiopicum* group *Gilo*) via Agrobacterium-mediated transformation. Genetically engineered plants exhibited high concentrations of anthocyanin in leaves, petals, stamens, and fruit peels under normal growth conditions, especially in fruit flesh. Furthermore, highly methylated anthocyanins, malvidin 3-(*p*-coumaroyl)rhamnoside(glucoside)-5-glucoside and malvidin 3-(feruloyl)rhamnoside(glucoside)-5-glucoside, were separated from the purple fruit flesh and identified by HPLC–ESI–MS/MS. qRT-PCR analysis revealed that most anthocyanin structural genes were dramatically up-regulated in the tissues of transgenic lines compared with non-transformed plants. In addition, the transgenic seedlings had greater tolerance to freezing stress and better recovery under rewarming conditions. These results provide a good foundation for the breeding of new eggplant cultivars with more healthy agronomic traits in future studies.

Keywords Eggplant · *SmMYB1* · Anthocyanin accumulation · HPLC–ESI–MS/MS · Transcriptional regulation · Freezing stress

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Abbreviations
PAL	Phenylalanine ammonia lyase
C4H	Cinnamate 4-hydroxylase
4CL	4-Coumarate-CoA ligase
CHS	Chalcone synthase
CHI	Chalcone isomerase
F3H	Flavone 3-hydroxylase

| F3′H	Flavonoid 3′-hydroxylase
DFR	Dihydroflavonol reductase
ANS	Anthocyanidin synthase
UFGT	Flavonoid-3-O-glucosyltransferase
GST	Glutathione S-transferase
OMT	O-Methyltransferase
DHK	Dihydrokaempferol
DHQ	Dihydroquercetin
DHM	Dihydromyricetin
HPLC	High-performance liquid chromatography
ESI–MS/MS	Electrospray ionization tandem mass spectrometry
qRT-PCR	Quantitative real-time PCR

Introduction

Anthocyanins, an important subgroup of flavonoids, are the main natural water-soluble pigments widely distributed in the plant kingdom. They are responsible for the red, blue, and purple colors found in plant tissues in a wide range of flowers, fruits, foliage, seeds, and roots (Koes et al. 2005; Tanaka et al. 2008). Large-scale studies indicate that the colorful pigments furnish flowers and fruits with eye-catching colors to attract insects, birds, and animals for pollination and seed dispersal (Bradshaw and Schemske 2003). Anthocyanins also play important roles in protecting plants against damage from UV radiation, freezing stress, drought stress, and microbial agents (Grote-wold 2006; Harborne and Williams 2000; Mendez et al. 1999). Earlier studies proved that regular intake of foods rich in anthocyanins could reduce the risks of suffering from chronic illnesses such as cancer, atherosclerosis, and degenerative disease (de Pascual-Teresa and Sanchez-Ballesta 2007; Espley et al. 2013). Vegetables, grains, and fruits rich in anthocyanins are therefore receiving more and more attention from consumers.

To date, anthocyanin biosynthesis has been studied extensively at the metabolic and molecular levels (Harborne and Williams 2000). Most of the structural genes encoding the enzymes responsible for each step have been isolated from various plants, for example Arabidopsis (Arabidopsis thaliana), apple (Malus ×domestica), petunia (Petunia hybrida), grape (Vitis vinifera), and other species (Honda et al. 2002; Shi and

Xie 2014; Tsuda et al. 2000; Van der Krol et al. 1990). As Fig. 1 shows, in the multienzymatic pathway, the initial precursor phenylalanine is catalyzed stepwise by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase (F3′5′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and O-methyltransferase (OMT) (Li et al. 2006). The colorful anthocyanindins are then catalyzed by flavonoid-3-O-glucosyltransferase (UFGT) for glycosylation and form more stable molecules, anthocyanins (Jaakola 2013; Winkel-Shirley 2001). In addition, dihydrokaempferol can be hydroxylated by F3′H or F3′5′H to produce dihydroquercetin and dihydromyricetin, respectively. DFR and the subsequent enzymes of the anthocyanin pathway will then catalyze dihydrokaempferol, dihydroquercetin, and dihydromyricetin to produce of the brick–red pelargonidin, red cyanidin, and violet delphinidin compounds, respectively. However, cyanidin and delphinidin can be further methylated at positions 3′ and/or 5′ on the B ring, eventually leading to the production of peonidin, petunidin, or malvidin.

Recently, a large number of studies have shown that the anthocyanin biosynthesis genes are regulated mainly at the mRNA level by transcription factors (TF) of several families. The TFs are within the MADS box, MYB, basic helix–loop–helix (bHLH), and WD40 repeat protein families (Goff et al. 1992; Jaakola 2013; Koes et al. 2005; Lalusin et al. 2006). A number of anthocyanin biosynthesis regulatory genes have been cloned from various plants, such as Arabidopsis, petunia, maize, apple, tomato, and other species (Ban et al. 2007; Bernhardt et al. 2005; Borevitz et al. 2000; Chiu et al. 2010; Jaakola et al. 2010; Lalusin et al. 2006; Mathews 2003; Morita et al. 2006; Park et al. 2007; Schwinn et al. 2006; Spelt et al. 2000; Takos et al. 2006). By analyzing mutants with abnormal levels of anthocyanins in model plants with genetic and molecular technology, it has been proved that the transcription of anthocyanin biosynthesis genes is directly regulated by a transcriptional activation of the MYB–bHLH–WD40 complex (MBW) that consists of R2R3 MYB, Bhlh, and WD40 proteins (Albert et al. 2011; Baudry et al. 2004; Broun 2005; Gonzalez et al. 2008; Lepiniec et al. 2006). However, MADS box genes probably function on the upstream

Fig. 1 Schematic representation of the biosynthetic pathway of the anthocyanins. The names of the compounds are indicated in *boxes*. The enzyme names are *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* flavanone 3′-hydroxylase, *F3′5′H* flavanone 3′,5′-hydroxylase, *DFR* dihydroflavonol 4-reductase, *ANS* anthocyanidin synthase, and *OMT* O-methyltransferase
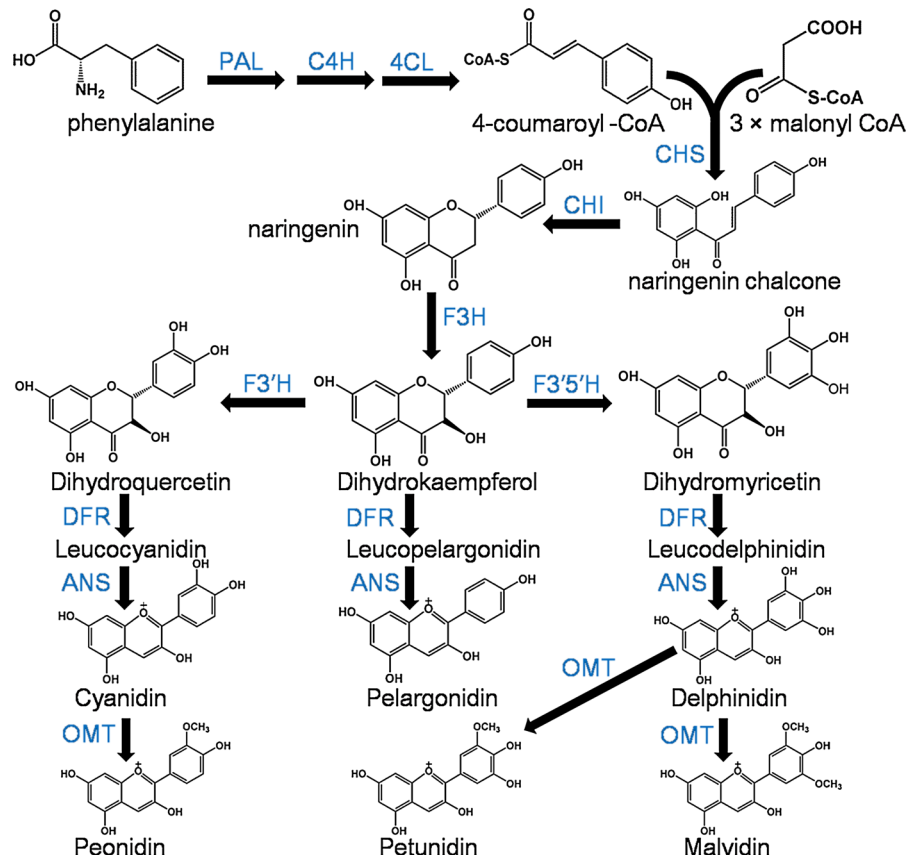
pathway of MYB proteins in bilberry (Jaakola et al. 2010). Previous studies showed that purple leaves or fruits rich in anthocyanin arise from the transcriptional activation of MYB transcription factors (Ban et al. 2007; Chiu et al. 2010; Mano et al. 2007; Mathews 2003; Takos et al. 2006). These studies centered on the molecular analysis of anthocyanin biosynthesis provide essential knowledge for technologists to genetically enhance the anthocyanin contents of crops and vegetables (Butelli et al. 2008; Gonzali et al. 2009).

In previous study, we reported that the *SmMYB1* gene encoding a R2R3 MYB transcription factor participated in the process of anthocyanin biosynthesis regulation in the peel of purple eggplant cultivar (*Solanum melongena* L) (Zhang et al. 2014c). A binary vector containing the full-length coding sequence of *SmMYB1* driven by the 35S promoter was then created. In this article, a non-anthocyanin-accumulating eggplant cultivar (*Solanum aethiopicum* group *Gilo*) with medicinal and ornamental value was used for transformation via Agrobacterium-mediated T-DNA transfer (Toppino et al. 2008). The

transgenic eggplants showed intense purple pigments in most tissues, especially in the flesh of tender fruits. Anthocyanins in the sarcocarp of transgenic eggplant were separated and identified by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS). Furthermore, the expression of anthocyanin biosynthesis and regulatory genes was analyzed in the tissues which displayed the accumulation of purple pigments. In addition, the freezing stress tolerance of transgenic and wild-type seedlings was analyzed.

Materials and methods

Plant materials and growth conditions

The seeds of eggplant (*Solanum aethiopicum* group *Gilo*) were obtained from Chongqing Academy of Agriculture Sciences. Transgenic and wild-type seedlings were grown in the greenhouse with 16-h/8-h

light/dark photoperiod (28 °C) and watered daily. The leaves, petals, stamens, peels, and flesh were collected, immediately frozen with liquid nitrogen, and stored at −80 °C for RNA extraction and other analyses. In addition, the peels and flesh of tender fruits (immature fruits) were collected at 15 days after anthesis.

Vector construction and plant transformation

The full-length open reading frame of *SmMYB1* (GenBank: KF727476) was amplified using primers SmMYB1-con-F, 5′-GCGATCTAGAAATAAAATG AATAATCCTCC-3′, and SmMYB1-con-R, 5′-GACT GAGCTCCGAAAACAGACAATATTACTA-3′. The PCR products were linked into plasmid (pBI121) to yield an overexpression plasmid pBI121-SmMYB1, containing the *SmMYB1* cDNA driven by the 35S promoter. The construct was then transferred into *Agrobacterium tumefaciens* strain LBA4404 and used to infect eggplant cotyledon explants (Filippone and Lurquin 1989; Zhang et al. 2014c). Transformed lines were selected for kanamycin (80 mg L^{-1} final concentration) resistance and then confirmed by PCR using the primers NPTII-F/R (NPTII-F GACAATCGGCTGCT CTGA and NPTII-R AACTCCAGCATGAGATCC).

RNA extraction and quantitative real-time PCR analysis

Total RNA of all samples was isolated using RNAiso Plus (TaKaRa, Dalian, PRC) according to the manufacturer's instructions. Then, 1 μg total RNA was used as a template for reverse transcription PCR with an oligo(dT)$_{20}$ primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's protocol. The synthesized cDNAs were diluted twice in RNase-/DNase-free water for qRT-PCR analysis.

qRT-PCR was performed using the CFX96^{TM} Real-Time System (C1000^{TM} Thermal Cycler, Bio-Rad). The qRT-PCR mixture (10 μL of total volume) consisted of 5 μL of GoTaq® qRT-PCR Master Mix (Promega), 0.5 μL of mixed primers, 1 μL of diluted cDNA, and 3.5 μL of RNase-/DNase-free water. PCR amplification was carried out using two-step cycling conditions of 98 °C for 3 min, followed by 40 cycles of 98 °C for 2 s and 60 °C for 10 s. NRT (no reverse transcription control) and NTC (no template control) were performed as negative controls. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 60–95 °C melts. Melting curve analysis of qRT-PCR samples revealed that there was only one product for each gene primer reaction. Three replicates for each sample were used, and *SmGAPDH* was used as internal standard. The nucleotide sequences of primer pairs used for qRT-PCR analysis are listed in Supplementary Table 1 (Zhang et al. 2014c).

Chemicals

The external standards, anthocyanin (delphinidin 3-rutinoside chloride and malvidin 3-glucoside chloride), were purchased from Phytolab (Germany). The HPLC-grade formic acid and methanol (MeOH) were bought from Sigma. All of the other solvents were provided by Aldrich (St. Louis, MO, USA).

Table 1 Anthocyanin levels (mg/g dry weight) in the purple fruit flesh of transgenic eggplant ($n = 3$)

No.[a]	RT[b] (min)	[M+M]$^+$ (m/z)	MS/MS (m/z)	Anthocyanin	Content
1	7.40	919	757/465/303	Delphinidin 3-(p-coumaroyl)rhamnoside(glucoside)-5-glucoside	0.013 ± 0.002
2	9.62	949	787/479/317	Petunidin 3-(rutinoside)glucoside-5-glucoside	0.029 ± 0.003
3	12.01	933	771/479/287	Cyanidin 3-(p-coumaroyl)rhamnoside(glucoside)-5-glucoside	0.012 ± 0.001
4	13.09	963	801/479/317	Petunidin 3-(feruloyl)rhamnoside(glucoside)-5-glucoside	0.069 ± 0.012
5	22.94	977	815/493/331	Malvidin 3-(feruloyl)rhamnoside(glucoside)-5-glucoside	0.272 ± 0.043
6	26.31	947	785/493/331	Malvidin 3-(p-coumaroyl)rhamnoside(glucoside)-5-glucoside	1.373 ± 0.192
7	29.59	Un	303/789	Delphinidin-based anthocyanin	0.258 ± 0.033
Total					2.026 ± 0.286

[a] *No.* corresponds to the elution order by HPLC analysis in Fig. 4a

[b] *RT* is the retention time. *Un* indicates that the molecular weight of the compound in reference to peak 7 is uncertain at present

Anthocyanin extraction and HPLC–ESI–MS/MS analysis

Anthocyanins were extracted and separated by HPLC according to the protocols described by Park and others with minor modifications (Park et al. 2011; Zhang et al. 2014c). Each sample (100 mg) was extracted with 1 mL of methanol/H_2O/acetic acid (85:15:0.5) at room temperature. The solution was vigorously vortexed for 5 min, sonicated for 5 min, and then centrifuged at 12,000g for 8 min. The extraction for each sample was performed twice, and the supernatants were mixed for the following HPLC analysis. The extract was filtered through a 0.2-μm PTFE syringe filter to remove cell debris. The filtrate was analyzed by an Agilent Technologies 1200 Series HPLC (Agilent Technologies, Palo Alto, CA, USA), equipped with an Agilent 1200 HPLC variable wavelength detector. The data were analyzed by Agilent 1200 HPLC ChemStation software. The chromatographic separation was carry out on a Zorbax Stablebond Analytical SB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent Technologies, Rising Sun, MD, USA). The injection sling was 10 μL. Elution was performed using mobile phase A (aqueous 5 % formic acid solution) and mobile phase B (methanol). The detection was set at a wavelength of 520 nm, and the column oven temperature was set at 40 °C. The flow rate was 1 mL/min. The gradient program was as follows: 0–5 min, 10–20 % B; 5–10 min, 20–30 % B; 10–15 min, 30–40 % B; 15–25 min, 40 % B; 25–30 min, 40–60 % B; 31–32 min, 90 % B; and 32–35 min, 10 % B. Quantification of the different anthocyanins was based on peak areas and calculated as equivalents of the external standards. All contents were expressed as milligrams per gram of dry weight. Low-resolution electrospray mass spectrometry was performed with a solariX ion trap mass spectrometer (Bruker Daltoniks, Billerica, MA, USA). The experimental conditions were as follows: ESI interface, nebulizer, 50 psi; dry gas, 15.0 psi; dry temperature, 320 °C; MS/MS, scan from m/z 200 to 2000; ion trap, scan from m/z 200 to 2000; source accumulation, 50 ms; ion accumulation time, 300 ms; flight time to acq. cell, 1 ms; smart parameter setting (SPS), compound stability, 50 %; and trap drive level, 60 %.

Total anthocyanin content analysis

The total anthocyanin contents were measured by a pH-differential spectrum method (Park et al. 2011). Frozen samples (100 mg) were crushed into powder in liquid nitrogen and then treated separately with 2 mL of pH 1.0 buffer (50 mM KCl and 150 mM HCl) and 2 mL of pH 4.5 buffer (400 mM sodium acetate and 240 mM HCl). The solution was vigorously vortexed for 10 min, sonicated for 10 min, and placed at 4 °C for 12 h. The mixtures were centrifuged at 12,000g for 20 min at 4 °C. Absorbance of the supernatants was then measured at 510 nm. The total anthocyanin content was calculated according to the equation:

$$\text{Amount} \left[\text{mg g}^{-1}(\text{FW}) \right] = (A1 - A2) \times 484.8/24825 \times \text{dilution factor},$$

where $A1$ represents the absorbance of supernatants gathered from pH 1.0 buffer solution at 510 nm, and $A2$ represents the absorbance of supernatants gathered from pH 4.5 buffer solution at 510 nm. The value 484.8 represents the molecular mass of cyanidin-3-glucoside chloride, while 24,825 reflects its molar absorptivity at 510 nm. Each sample was analyzed in triplicate, and the results were presented as the average of the three measurements.

Dehydration assay of leaves of transgenic and control plants

For the dehydration assay of leaves, 35-day-old seedlings of wild-type and transgenic plants were harvested for analysis. Leaves of a similar size, age, and position were detached and placed on dry filter papers for the measurement of the water loss rate. The leaves were weighed at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 16 h and then placed in the oven until constant weight. Water loss of leaf was presented as a percentage of initial fresh weight.

Freezing tolerance assay of transgenic and control plants

For the freezing treatment, 35-day-old seedlings of wild-type and transgenic plants were incubated at

-5 ± 1 °C for 0 h, 2 h, 4 h, and 8 h. The leaves were then harvested for analysis. Twenty leaf disks (each 1 cm^2) were placed in a transparent container containing 15 ml deionized water and shaked for 2 h before measurement of the initial electric conductance ($S1$). The transparent container was then heated in boiling water for 20 min and cooled to room temperature to measure the ultimate electric conductance ($S2$). The electric conductance of deionized water was used as the blank ($S0$) (Rapisarda et al. 2000). The relative electrolyte leakage (REL) was calculated from the following equation:

$$REL\ (\%) = (S1 - S0)/(S2 - S0) \times 100$$

Statistical analyses

SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for the data analysis. One-way analysis of variance (ANOVA) was performed with post hoc Tukey's honestly significant difference (HSD) test, with significance set at $p < 0.05$ and $p < 0.01$.

Results

SmMYB1 ubiquitously promotes anthocyanin biosynthesis in eggplant

To obtain transgenic eggplant with high levels of anthocyanin accumulation, a construction containing the full-length coding sequence of *SmMYB1* driven by the 35S promoter was created and transformed into wild-type eggplant (*Solanum aethiopicum* group *Gilo*) via *Agrobacterium tumefaciens*-mediated T-DNA transfer (Supplementary Figure 1A). Eight independent kanamycin-resistant seedlings were generated and were confirmed as transgenic eggplant by PCR. Three independent transgenic lines (lines N, P, and R) exhibiting extensive production of purple pigments in the whole plants were selected for further characterization. The transgenic plants showed pigmented leaves, stems, flowers, fruit peels, and flesh with prominent purple or violet colors (Fig. 2; Supplementary Figure 1). In addition, the pigments in the dark purple fruit of transgenic lines gradually faded as the process of ripening starts (data not shown). The phenotypes referred to above were also confirmed in T1 and T2 generations of transgenic eggplant plants.

Identification and quantitative analyses of the purple pigments in the fruit flesh of transgenic eggplant

A total of seven different anthocyanins were separated and characterized from the extracts of the purple fruit flesh of transgenic eggplant using the HPLC–ESI–MS/MS method (Fig. 3). For the purpose of verifying the identity of anthocyanins in transgenic eggplant, we analyzed the fragmentation patterns of MS/MS (m/z) of the compounds displayed in HPLC profiles with reference to the information of radical groups reported previously (Supplementary Figure 2A; Table 1). Consequently, seven different anthocyanins were identified as the major ingredients of the purple pigments in the fruit flesh of transgenic eggplant (Table 1). It is worthy of note that most of the anthocyanins identified were delphinidin and delphinidin derivative-based pigments. Highly methylated delphinidin-based anthocyanins, malvidin-based pigments, malvidin 3-(feruloyl)rhamnoside(glucoside)-5-glucoside, and malvidin 3-(p-coumaroyl)rhamnoside(glucoside)-5-glucoside occupied 13 and 68 % of the total amount of anthocyanins, respectively. However, it is strange that only trace amount of the cyanidin-based anthocyanin, cyanidin 3-(p-coumaroyl)rhamnoside(glucoside)-5-glucoside (0.012 mg/g dry weight), in reference to peak 3 were detected in the purple flesh (Fig. 3; Table 1). Moreover, pelargonidin-based anthocyanins were not detected in the corresponding tissue in transgenic eggplant.

Total content of anthocyanins in fruit flesh was found to be 2.026 mg/g dry weight using HPLC (Table 1), while anthocyanins were absent in the corresponding tissue of wild-type plants. However, the total content of anthocyanins in the purple flesh of transgenic eggplant was not as high as that found in fruits of transgenic tomato with fruit-specific expression of two anthocyanin biosynthesis regulators (*ROSEA* and *DEL*) (Butelli et al. 2008). The methylated anthocyanin, malvidin 3-(p-coumaroyl)rhamnoside(glucoside)-5-glucoside in reference to peak 6 (Fig. 3a, c), showed the highest level (1.373 mg/g dry weight) in the flesh of transgenic eggplant (Table 1), while the content of malvidin 3-(feruloyl)rhamnoside(glucoside)-5-glucoside in reference to peak 5 (Fig. 3a, b) ranked second.
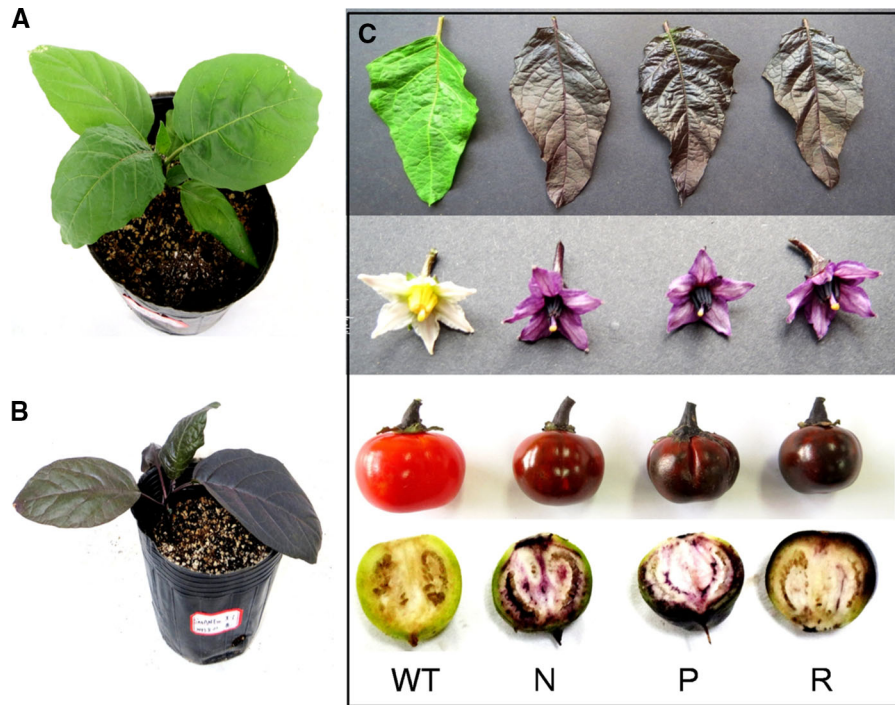
Fig. 2 Overexpression of *SmMYB1* promotes ubiquitous anthocyanin accumulation in transgenic eggplants. *N*, *P*, and *R* indicate T1 progeny of independent transgenic lines transformed with binary vector pBI121-SmMYB1, while *WT* indicates non-transformed plants. **a** The 35-day-old eggplant seedlings of non-transformed plants. **b** The 35-day-old eggplant seedling of transgenic lines. **c** Anthocyanin accumulates ubiquitously in leaves, petals, stamen, fruit peels, and fruit flesh of transgenic eggplants with WT as control
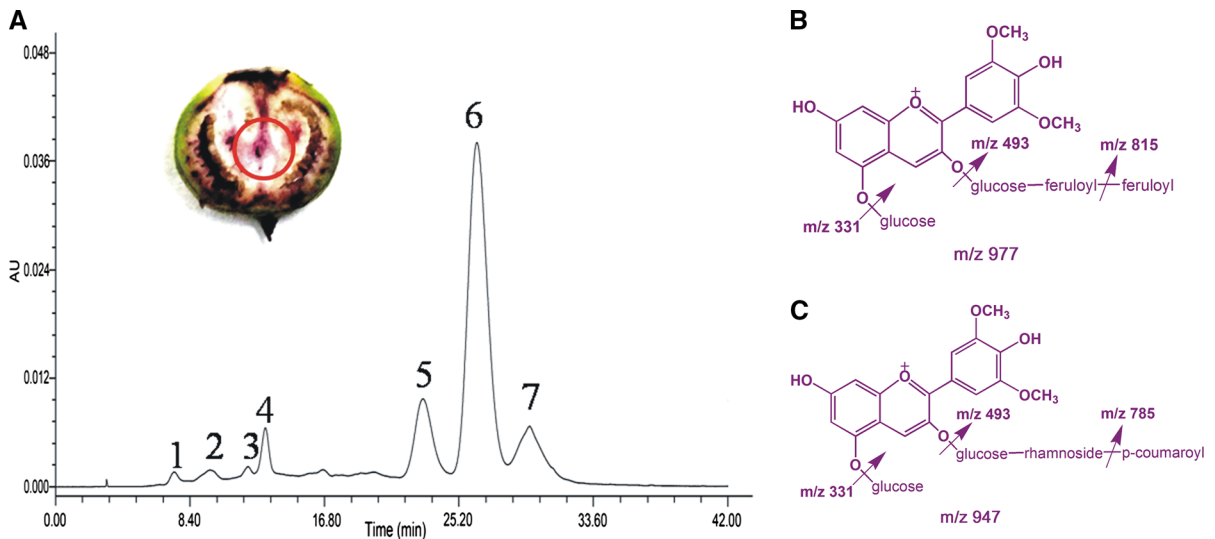

Fig. 3 Identification of anthocyanins in the purple fruit flesh of transgenic eggplants. **a** HPLC profiles of anthocyanins of the purple fruit flesh. The purple fruit flesh framed by the *red circle* in the *upper part* indicates the tissues collected for HPLC–ESI–MS/MS analysis. *Peak numbers* refer to the anthocyanins listed in Table 1. **b** Structure and major cleavage of malvidin 3-(feruloyl)rhamnoside(glucoside)-5-glucoside in reference to peak 5 of Fig. 3a. **c** Structure and major cleavage of malvidin 3-(p-coumaroyl)rhamnoside(glucoside)-5-glucoside in reference to peak 6 of Fig. 3a. (Color figure online)

Total anthocyanin contents of various tissues
in wild-type and transgenic eggplants

The anthocyanin contents of leaves, petals, stamens,
fruit peels, and flesh in wild-type and transgenic lines
were measured with a pH-differential spectrum
method. Almost no anthocyanins were detected in
various tissues of wild-type plants, while large amount
of anthocyanins were extracted from leaves, petals,
stamens, fruit peels, and flesh of transgenic lines. As
shown in Supplementary Figure 3A, the highest level
of anthocyanin accumulation was 2.26 mg/g fresh
weight in the stamens of transgenic line P, while the
total anthocyanin contents of petals and leaves ranked
second and third, respectively. It is worth noting that
considerable amounts of anthocyanin accumulation
were also detected in fruit peels and flesh of transgenic
eggplants (Supplementary Figure 3A).

Transcriptional analysis of *SmMYB1* gene in wild-
type and transgenic eggplants

To investigate the intrinsic molecular mechanisms
underlying the specific pattern of anthocyanin accu-
mulation, the expression profile of *SmMYB1* and total
contents of anthocyanins were analyzed in leaves,
petals, stamens, fruit peels, and flesh of wild-type and
transgenic eggplants. Supplementary Figure 3A
shows that anthocyanins accumulate ubiquitously at
high levels in transgenic eggplants compared with the
background value in non-transformed eggplants.
Supplementary Figure 3B shows the dramatically
high expression of *SmMYB1* in various plant tissues
of transgenic lines compared with wild-type eggplant.
However, the times of increase vary in different tissues
of transgenic lines. In leaves, stamens, and flesh,
SmMYB1 was up-regulated more than 1000-fold,
while *SmMYB1* was only up-regulated 50-fold in
peels. In addition, the times of increase of *SmMYB1*
gene were found to be merely 4–5-fold in transgenic
petals. It is worth mentioning that the times of increase
depend on the background levels of *SmMYB1* in
different tissues of wild-type eggplant. However, the
total contents of anthocyanins were closely in accor-
dance with the expression levels of the *SmMYB1* gene
in various tissues of transgenic eggplant. These results
demonstrate that overexpression of *SmMYB1* pro-
motes abundant anthocyanin biosynthesis ubiqui-
tously in eggplant (*Solanum aethiopicum* group *Gilo*).

Expression profiles of anthocyanin biosynthesis
genes in various tissues of wild-type
and transgenic eggplant

In the earlier study, we showed that SmMYB1 lies in the
same cluster as the other anthocyanin synthesis activa-
tors from various species and SmMYB1 could stimulate
anthocyanin accumulation in kanamycin-resistant callus.
To further study the molecular mechanisms underlying
anthocyanin accumulation in transgenic eggplants, we
investigated the expression profiles of anthocyanin
structural genes *SmPAL*, *SmCHS*, *SmCHI*, *SmF3H*,
SmF3′5′H, *SmDFR*, *SmANS*, and *SmUFGT* in leaves,
petals, stamens, fruit peels, and flesh between wild-type
and transgenic eggplant (Supplementary Figure 4A–H).
Overall, these results showed that the expression levels
of all structural genes, except *SmPAL* and *SmCHI*, were
significantly up-regulated in different transgenic egg-
plant tissues compared with wild-type eggplant.

In detail, the expression levels of late structural
genes *SmF3′H*, *SmF3′5′H*, *SmDFR*, *SmANS*, and
SmUFGT were all dramatically up-regulated in trans-
genic tissues, especially in fruit flesh (Supplementary
Figure 4D, E, F, G), whereas of the early structural
genes, only *SmCHS* was significantly up-regulated in
various transgenic tissues, with the exception of
stamens. In the fruit flesh of transgenic lines, *SmCHI*
was also up-regulated to a level which is comparable to
the expression in other tissues. It is noteworthy that
most structural genes showed the highest expression
levels in petal and stamen tissues of wild-type plants.
Therefore, the times of increase of these genes in
transgenic petal and stamen tissues were limited. Even
so, most late structural genes showed the highest levels
of transcripts in petal and stamen tissues of transgenic
lines. Thus, it can be seen that the expression profiles of
most structural genes coincide well with the expression
pattern of *SmMYB1*. Therefore, all the results men-
tioned above prove that the overexpression of tran-
scription factor SmMYB1 in transgenic eggplants
promotes anthocyanin accumulation by transcriptional
activation of most anthocyanin biosynthesis genes.

Expression profiles of anthocyanin biosynthesis
regulatory genes in various tissues of wild-type
and transgenic eggplants

To investigate the mechanisms underlying the tran-
scriptional regulation of structural genes, the

expression levels of transcriptional factors *SmbHLH1* and *SmAN11* were analyzed in various tissues of wild-type and transgenic lines by qRT-PCR. Compared with the wild type, the expression of *SmbHLH1* and *SmAN11* both remained stable in various tissues of the transgenic lines, except in stamens (Supplementary Figure 5A and B). In the highly complicated regulatory network of anthocyanin biosynthesis, R2R3 MYB proteins participate in the transcriptional activation of the AtTT8 homologs and promote pigment production coordinately with bHLH proteins in many species (Butelli et al. 2008; Chiu et al. 2010; Yuan et al. 2009; Zhang et al. 2015). However, it is evident that the suppressed expression of *SmbHLH1* did not arise directly from the overexpression of *SmMYB1* in transgenic stamens. Similarity and phylogenetic analysis of SmbHLH1 with other bHLH proteins from various species suggests that SmbHLH1 should be a functional anthocyanin biosynthesis activator in eggplant. Here, we inferred that the decreased expression of *SmbHLH1* for unknown reasons might be responsible for the down-regulation of *SmPAL* and *SmCHS* in transgenic stamens compared with non-transformed plants, whereas the slightly increase in *SmAN11* indicates that the *WD40* gene is induced by *SmMYB1* in a non-stringent manner.

Overexpression of *SmMYB1* gene enhances tolerance to freezing stress

The production of anthocyanins in vegetative tissues often increases the tolerance of plants to various environmental stressors (Holton and Cornish 1995; Klaper et al. 1996; Yakimchuk and Hoddinott 1994). To investigate whether high amounts of anthocyanins confer defense against freezing stress, the effects of freezing stress on the transgenic and non-transformed eggplants were tested in 35-day-old seedlings. No significant differences in growth rates were observed between the transgenic plants and wild-type plants cultured at 25 °C up to 35 days (Fig. 4a). By examining the dry weight of leaves at the same developmental stage, the results showed that there were no obvious differences between transgenic and wild-type plants (data not shown). However, we checked the detached-leaf water loss rate, and the results showed that the leaves from the transgenic plants had lower water loss rates than wild-type plants at each dehydration time point (Fig. 4d).

For the freezing treatment, wild-type and transgenic plants of 35 days were incubated at -5 ± 1 °C for 0 h, 2 h, 4 h, and 8 h. After incubation under freezing stress for 8 h, all the leaves of the plants were injured, but the leaves of wild-type plants were injured much more seriously than those of the transgenic lines (Fig. 4b). In addition, the transgenic plants showed a good recovery upon rewarming conditions, while the wild-type plants eventually died because of the serious damage in leaves and buds (Fig. 4c). To further study the mechanisms underlying the increased freezing tolerance, the electric conductance of leaves was measured at each time point in the freezing treatment experiment. Figure 4e shows that the relative electrolyte leakage (REL) increased markedly slower in transgenic plants than in wild-type plants. These results indicate that the cell membrane of transgenic leaves was less damaged by the freezing treatment.

Discussion

Eggplant (*Solanum melongena* L.), an important agronomical solanaceous crop, is cultivated worldwide for its tender fruits (immature fruits). It was ranked as one of the top ten vegetables for its capacity of oxygen radical scavenging due to phenolic constituents in the fruit (Cao et al. 1996). Eggplant cultivars differ in fruit size, color, and shape, but the cultivars with dark purple pigments in fruit skin are apparently more popular for their high nutritional value. However, it is a pity that to date no anthocyanins have been detected in the flesh of the cultivars grown all over the world. Therefore, we attempted to cultivate new purple-fleshed eggplant cultivars by genetic engineering.

In a previous article, a functional MYB transcription factor, *SmMYB1*, was isolated from the edible cultivar (Zi Chang) of eggplant. In this study, a wild eggplant cultivar (*Solanum aethiopicum* group *Gilo*) with no visible anthocyanins was used for transformation. Fortunately, transgenic plants with high expression levels of *SmMYB1* display abundant anthocyanin accumulation ubiquitously in various tissues including the flesh of fruits. Even stigmas also show light purple pigments at the top. These results suggest that anthocyanin production can in fact be induced in any tissues in wild eggplant.

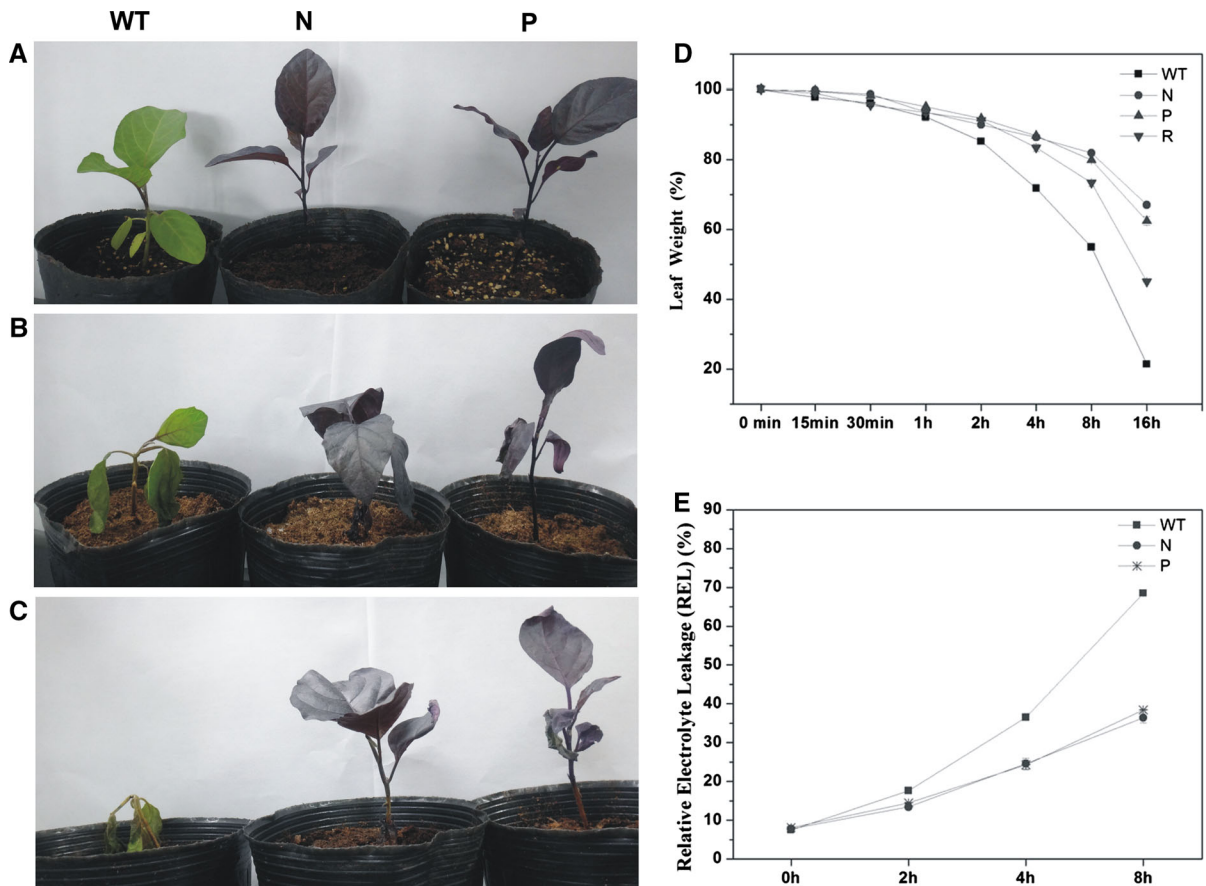By analyzing the purple pigments in flesh of transgenic eggplant with HPLC–ESI–MS/MS,

Fig. 4 The appearance of wild-type (*WT*) and transgenic seedlings of eggplants under freezing stress. **a** The 35-day-old eggplant seedlings of WT and transgenic lines grown at 25 °C. **b** The 35-day-old eggplant seedlings of WT and transgenic lines after 8-h freezing treatment (-5 ± 1 °C). **c** The 35-day-old eggplant seedlings of WT and transgenic lines under rewarming conditions. **d** The water loss rates of WT and transgenic plants. **e** The electric conductance of leaves at each freezing treatment time point. The individual plants shown are representative of the three plants grown per treatment

malvidin-based pigments were identified as the chief components of the separated anthocyanins. In the biosynthesis pathway of anthocyanin, flavonoid 3′,5′-hydroxylase catalyzed DHK to produce DHM at positions 3′ and 5′ in the B ring with extremely high efficiency. Then, in subsequent reactions, *O*-methyltransferase (OMT) catalyzes methylation at the 3′ and/or 5′ hydroxyl groups of most delphinidins to produce petunidin or malvidin-based anthocyanins, respectively. Combined with the expression analysis of anthocyanin structural genes in various tissues between wild-type and transgenic lines, it can be seen that the dramatic up-regulation of *SmF3′5′H* was responsible for the abundant production of delphinidin. Meanwhile, it is not strange that pelargonidin- and cyanidin-based anthocyanins were seldom detected in

the corresponding tissue in transgenic eggplant. Compared with the results previously reported, the anthocyanins identified in transgenic eggplant are evidently different from the pigments characterized in the non-transformed cultivars (Wu and Prior 2005). The anthocyanins were methylated in a great extent at the position of the B ring in fruit flesh of transgenic eggplants, whereas that is very rare in edible eggplant. Perhaps the high expression level of *SmMYB1* activated the transcription of some uncharacterized OMTs in transgenic eggplant. It can be seen that all the anthocyanins in transgenic eggplant were glycosylated pigments which arise from glycosylation at the C5 and C3 positions of anthocyanins, while the acylated form of modification at the C3 position of the C ring in anthocyanin precursors seems quite common in this

study. These results indicate that glycosylation, acylation, methylation, and hydroxylation of anthocyanin precursors in flavonoid metabolism were the main patterns of modification in fruit flesh of transgenic eggplant.

Although no visible anthocyanin pigmentation was apparently observed in the wild-type tissues studied above, a certain amount of transcripts of all the anthocyanin structural genes were detected in the corresponding tissues, especially in petals and stamens. These results indicate that adequate transcripts of most structural genes should be a prerequisite for large amounts of anthocyanin accumulation in various tissues of transgenic eggplant. This phenomenon seems quite common in many species which showed visible anthocyanin accumulation (Borevitz et al. 2000; Chiu et al. 2010; Mathews 2003; Yuan et al. 2009; Zhang et al. 2014b, 2015). Obviously, the threshold of structural genes for activating abundant anthocyanin production should be much higher than the expression levels in petals and stamens of non-transformed eggplant.

In the phenylpropanoid pathway, the transcripts of *SmPAL* were almost unaffected in transgenic tissues except for a visible decrease in stamens (Supplementary Figure 4A). In addition, the transcript levels of *SmCHS* were down-regulated by nearly 80 % in stamens, compared with those significantly up-regulated in leaves, petals, fruit peels, and flesh of transgenic lines (Supplementary Figure 4B). As we have mentioned above, the total anthocyanin contents in stamens were apparently higher than all the other tissues of the transgenic lines, so it seems that large amounts of pigment production might impact negatively on the normal metabolism and development of transgenic stamens. Therefore, the transcription of *SmPAL* and *SmCHS* might be adjusted to a lower level to accommodate the global metabolism in purple stamens, the so-called feedback effect (Baudry et al. 2006).

Expression analysis of anthocyanin structural and regulatory genes in various tissues between wild-type and transgenic lines indicates that most anthocyanin biosynthetic genes were up-regulated accordingly by the transcriptional activation of the MBW complex consisting of SmMYB1 and other interaction partners. R2R3 MYB TFs have been implicated in regulation of anthocyanin biosynthesis by activating the transcription of anthocyanin structural genes in many plants (Albert et al. 2011; Borevitz et al. 2000; Bovy 2002; Jung et al. 2009; Mathews 2003; Schwinn et al. 2006;

Shin et al. 2006; Takos et al. 2006). In addition, overexpression of bHLH transcription factor solely can result in efficient anthocyanin accumulation in transgenic tomato (Albert et al. 2011; Bovy 2002; Mooney et al. 1995; Xie et al. 2012; Zhang et al. 2014b). In addition, the expression levels of most structural genes are dramatically up-regulated in transgenic eggplant tissues, while the expression of *SmCHS* and *SmPAL* was evidently suppressed in transgenic stamens. This abnormal phenomenon suggests that excessive production of anthocyanins may be toxic for the normal development and growth of stamens. The plants probably adjusted the anthocyanin production by inhibiting the transcription of several structural genes in the stamens themselves. Moreover, the suppression of *SmCHS* and *SmPAL* might arise from the slight down-regulation of *SmbHLH1* for unknown reasons in transgenic stamens.

Previous studies indicate a close link between freezing stress and anthocyanin accumulation in plants (Ahmed et al. 2014; Bovy 2002; Hannah et al. 2006; Xie et al. 2012). The high content of anthocyanin confers better tolerance to chilling stress in seedlings. Heterologous expression of *LeAN2* induces anthocyanin accumulation and also enhances resistance to chilling stress in Arabidopsis (Meng et al. 2014). In this study, we investigated the tolerance of transgenic and wild-type seedlings of eggplant under freezing stress. The results showed that the transgenic seedlings were less injured than wild-type under freezing stress for 8 h. The results of the relative electrolyte leakage (REL) assay demonstrated that membrane damage was less serious in transgenic seedlings. The lower water loss rates also indicated that transgenic seedlings may possess better tolerance to stressors (Harborne and Williams 2000). In addition, transgenic seedlings have exerted better recovery under rewarming conditions. These results suggest that a high concentration of anthocyanins provides transgenic eggplant seedlings with more tolerance to freezing stress.

Colorful vegetables and fruits rich in anthocyanins are attracting increasing attention from consumers for their eye-catching colors and health-promoting effects. Many technologists are focusing on breeding new cultivars of vegetables with high contents of anthocyanins with plant biotechnology (Butelli et al. 2008; Zhang et al. 2014a). In this study, transgenic eggplants with purple-fleshed fruit were generated. However, the distribution of anthocyanins was not

uniform in the flesh, and the total content was not comparable to that of transgenic tomato engineered by the combined expression of an R2R3MYB protein and a bHLH protein from snap-dragon driven by the fruit-specific promoter E8, since the expression levels of most anthocyanin biosynthesis genes in fruit flesh are much lower than other tissues including fruit peels, stamens, and leaves. These results in this article show that overexpression of *SmMYB1* only was insufficient to drive the abundant biosynthesis of anthocyanin in fruit flesh. More efforts should be given to the creation of transgenic eggplant with more anthocyanin biosynthesis regulators driven by fruit-specific promoters. Nonetheless, we provide a good foundation for the generation of genome-modified eggplant with higher content of anthocyanins. Moreover, the transgenic eggplant seedlings displayed a greater tolerance to freezing stress than wild-type. In conclusion, this work provides important information for breeding vegetables with health-promoting ingredients and better resistance to abiotic stress.

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