J. Plant Biol. (2017) 60:93-101
DOI 10.1007/s12374-016-0199-9

ORIGINAL ARTICLE

The MYB Transcription Factor *StMYBA1* from Potato Requires Light to Activate Anthocyanin Biosynthesis in Transgenic Tobacco

Yuhui Liu[1,2], Li Wang[1,3], Junlian Zhang[1,4,*], Bin Yu[1,2], Jing Wang[5] and Di Wang[1,2]

[1]*Gansu Key Laboratory of Crop Improvement and Germplasm Enhancement/Gansu Provincial Key Laboratory of Aridland Crop Science, Gansu Agricultural University, Lanzhou 730070, People's Republic of China*
[2]*College of Agronomy, Gansu Agricultural University, Lanzhou 730070, People's Republic of China*
[3]*College of Life Science and Technology, Gansu Agricultural University, Lanzhou 730070 People's Republic of China*
[4]*College of Horticulture, Gansu Agricultural University, Lanzhou 730070, People's Republic of China*
[5]*College of Food Science and Engineering, Gansu Agricultural University, Lanzhou 730070, People's Republic of China*

Received: April 17, 2016 / Accepted: November 21, 2016
© Korean Society of Plant Biologists 2017

Abstract *StMYBA1* from potato tuber was transformed into tobacco (*Nicotiana tabacum*) with the CaMV 35S promoter to investigate whether it can regulate anthocyanin biosynthesis in tobacco plants. As a result, anthocyanin accumulation was induced by overexpressing *StMYBA1* in all tissues of transgenic tobacco lines. Several anthocyanin biosynthetic genes were highly expressed in leaves and roots of transgenic lines. In particular, the bHLH transcriptional partners *NtAN1a* and *NtAN1b*, especially *NtAN1b*, were up-regulated significantly in leaves and roots of *StMYBA1*-overexpressing tobacco. These results demonstrate that endogenous bHLH partners can be stimulated by overexpressing exogenous gene *StMYBA1* in tobacco, and the elevated expression levels of bHLH partners are essential for anthocyanin production in plant tissues. Applying the dark conditions to the tobacco plants overexpressing *StMYBA1* transiently, can down-regulate the expression levels of biosynthetic pathway genes and bHLH TFs, and subsequently reduce anthocyanin accumulation. We suggest that StMYBA1 can positively regulate anthocyanin biosynthesis in tobacco, and light is required for its function on anthocyanin accumulation.

Keywords: Anthocyanin accumulation, Light, Potato, *StMYBA1*, Tobacco

Introduction

A variety of secondary plant polyphenolic metabolites, such as flavones, flavonols, anthocyanins and proanthocyanidins is uniquely found in plants that known collectively as flavonoids. Flavonoids exert distinct effects on a variety of essential biological processes in plants, as well as in animals as they consuming flavonoids in their diet (Dubos et al. 2008). Nowadays, interest in these secondary metabolites has been grown rapidly due to their potential benefits for human health (Bazzano et al. 2003; Wang et al. 2009).

Anthocyanins, derived from the anthocyanin biosynthesis pathway, are the largest group of water-soluble plant flavonoids found in organs of plants and crops (Holton and Cornish 1995; Kim et al. 2003; Martens et al. 2010).

Anthocyanin biosynthesis and its regulation have been well studied. The anthocyanin biosynthetic genes are regulated transcriptionally by transcription factors (TFs) consisting of the MYB (mostly R2R3 MYB), bHLH and WD40 classes (Grotewold 2006; Allan et al. 2008). These TFs can form a ternary MYB-bHLH-WD40 (MBW) complex to regulate anthocyanin biosynthetic genes. In Arabidopsis, anthocyanin accumulation is transcriptionally controlled by the MBW complex consisting of one R2R3-MYB protein from PAP1 (AtMYB75), PAP2 (AtMYB90), MYB113, or MYB114, one bHLH protein from TRANSPARENT TESTA 8 (TT8), GLABROUS3 (GL3), or ENHANCER OF GLABRA3 (EGL3) and one WD40-repeat protein from transparent testa glabra1 (TTG1) (Gonzalez et al. 2008). Among these regulatory proteins, the MYB family contributes to the largest class of TFs, which can be categorized into four subfamilies - MYB1R, R2R3MYB, MYB3R, and MYB4R proteins according to one or more imperfect repeats which are highly conserved in the DNA-binding motif (Rosinski and Atchley 1998; Dubos et al. 2010).

The MYB subfamilies with two repeats - R2R3 MYB are

*Corresponding author; Junlian Zhang
Tel : +86-18919146885
E-mail : zhangjunlian77@163.com

the most common ones in plants, with 137 members of the R2R3 MYB have been identified, of which 13 members are considered to be associated with flavonoid metabolism (Dubos et al. 2010; Feller et al. 2011). The R2R3 MYB subfamilies play an important role in regulating the anthocyanin biosynthesis (Allan et al. 2008). The MYB *C1* of *Zea mays* has been identified as the first R2R3 MYB to control pigmentation (Paz-Ares et al. 1987), subsequently, the other MYB TFs modulating anthocyanin biosynthesis in other species such as Arabidopsis (Borevitz et al. 2000; Gonzalez et al. 2008), grape (Kobayashi et al. 2004), apple (Espley et al. 2007; Chagné et al. 2013), pepper (Borovsky et al. 2004), tomato (Mathews et al. 2003) and potato (Jung et al. 2009; Zhang et al. 2009) were identified.

Anthocyanin accumulation in various plant species is affected by a variety of environmental factors, such as light, nutrition, drought and temperature (Allan et al. 2008). Many studies have demonstrated that light has a decisive effect on pigment production in plants, resulting in the anthocyanin accumulation. On the contrary, the expressions of anthocyanin pathway genes were significantly down-regulated or repressed under weak light or dark conditions, resulting in decreased or no accumulation of anthocyanin in plants and crops (Islam et al. 2005; Rowan et al. 2009; Maier and Hoecker 2015).

Many studies have been performed in potato to explore regulatory mechanism of anthocyanin biosynthesis in tuber periderm (Jung et al. 2009; Payyavula et al. 2013), tuber flesh (Zhang et al. 2009) and leaves (D'Amelia et al. 2014). Jung et al. (2009) reported that an additional MYB with high homology to StAN1 protein, StMYBA1, is a possible StAN1 pseudogene corresponding to the published StAN3 sequence. but the function of StMYBA1 is not well studied. In the present study, regulation mechanism of anthocyanin biosynthesis in transgenic tobacco overexpressing potato MYB TF StMYBA1 has been further investigated based on the previous transient assay result (Liu et al. 2016). The result showed that anthocyanin synthesized by overexpressing *StMYBA1* in tobacco plants via regulating expression of the anthocyanin structural genes as well as endogenous bHLH TFs, which suggest that the elevated expression levels of bHLH partners are essential for anthocyanin production in plant tissues. Our results also showed that the light plays an essential role in regulating anthocyanin pathway genes and bHLH TFs as well as the anthocyanin accumulation, suggesting the other light-regulated factors may be involved.

Results

Phenotype of Transgenic Plants

Kan-resistant shoots were regenerated from the cut surface of inoculated leaf explants on MS medium with kanamycin and Timentin after six or eight weeks. Roots were then induced from these shoots after 1 month of culture. Three individual transgenic tobacco lines were confirmed by real-

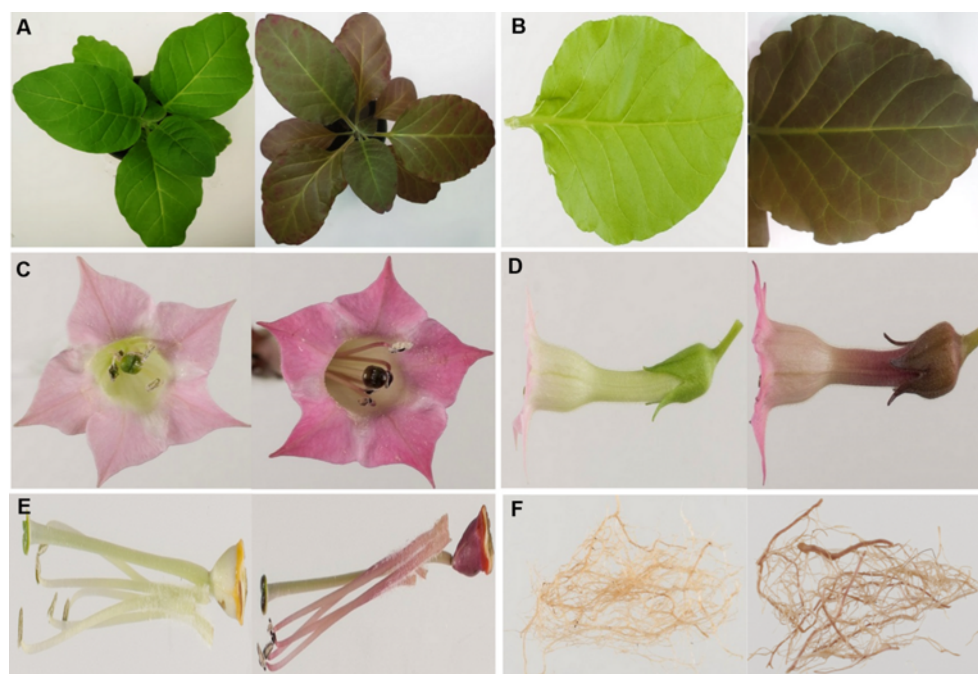

Fig. 1. Phenotype characteristics of transgenic tobacco plants overexpressing *StMYBA1* (on the right) and empty vector as control (on the left). (A) Whole plant, (B) Leaf, (C) Flower, (D) Sepal, (E) Anther and Ovary, (F) Root

time qPCR analysis. Compared to the control plants transformed with empty vector, enhanced red pigment was accumulated in leaves, flowers, ovaries and roots of the *StMYBA1*-overexpressing transgenic tobacco lines (Fig. 1).

StMYBA1 Activates Most of the Anthocyanin Pathway Genes in Leaves and Roots of Transformed Tobacco Lines

The expression levels of anthocyanin pathway genes in leaves and roots of transgenic tobaccos were examined to further investigate the efficiencies of StMYBA1 in inducing anthocyanin biosynthesis.

StMYBA1 was overexpressed and anthocyanin was induced in all leaves and roots of three transgenic tobacco lines (Fig. 2A, B), of which, the highest anthocyanin was accumulated in transgenic line 2, which induced 126.02±10.71 mg 100 g^{-1} FW and 46.02±8.24 mg 100 g^{-1} FW of anthocyanin in leaves and roots, respectively (Fig. 2B), while no anthocyanin was detected in control tobacco plants carrying empty vector.

Compared to the control tobacco plants, the expression levels of several anthocyanin biosynthetic genes such as *NtCHI*, *NtCHS*, *NtF3H*, *NtF3'H*, *NtDFR*, *NtANS* and *NtUFGT*, were up-regulated in leaves and roots of the three transgenic tobacco lines (Fig. 3). *NtANS* was highly up-regulated in leaves and *NtDFR* was most strongly up-regulated in roots of transgenic plants. These results indicated that *StMYBA1* transformation enhanced anthocyanin accumulation significantly in transgenic tobacco.

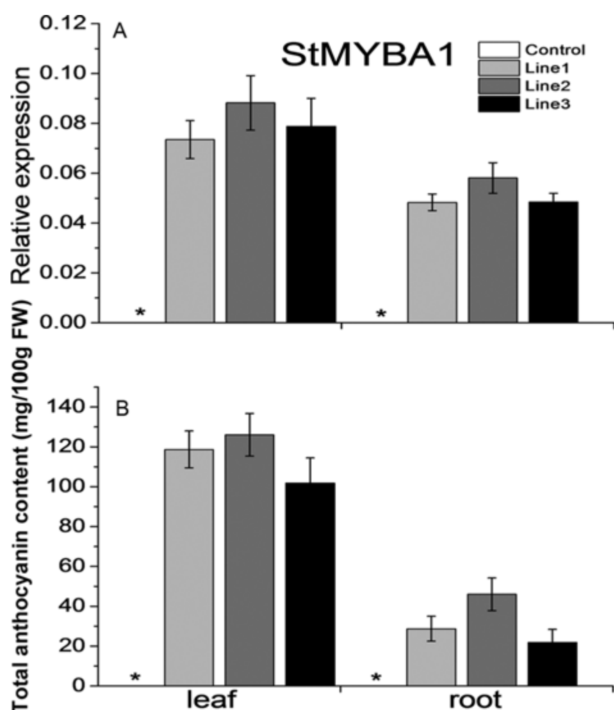

Fig. 2. Real-time qPCR analysis of expression levels of *StMYBA1* and total anthocyanin content in leaves and roots of transgenic tobacco plants overexpressing *StMYBA1*. (A) *StMYBA1* expression in leaf and root, (B) total anthocyanin content in leaves and roots. The data represent the means ± SE of four technical replicates. Genotypes denoted by *showed that no anthocyanin and expression were detected in this study

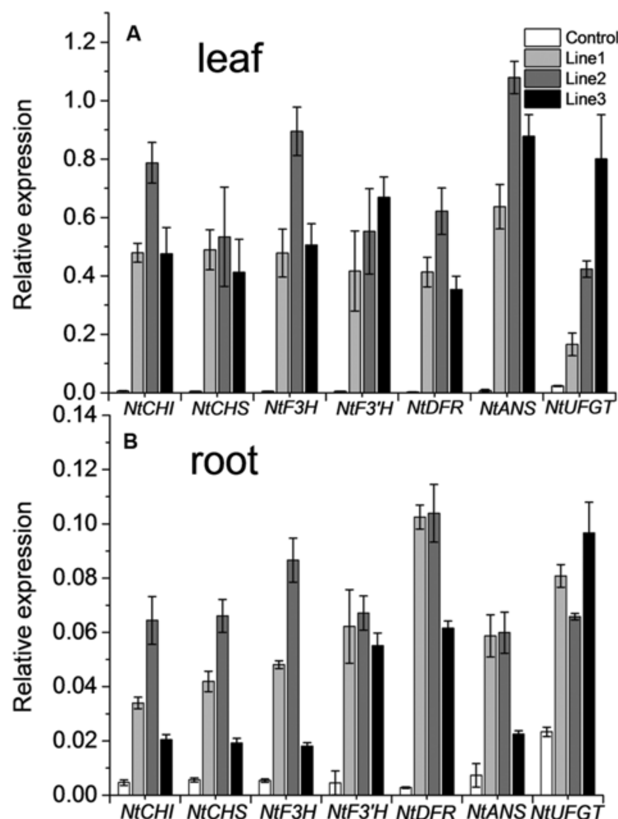

Fig. 3. Real-time qPCR analysis of expression levels of anthocyanin biosynthetic genes in leaves (A) and roots (B) of transgenic tobacco plants overexpressing *StMYBA1*. The data represent the means ± SE of four technical replicates

Over-expression of *StMYBA1* Activates the Expression of Endogenous bHLHs in Transgenic Tobacco

The expression of *NtAN1a* and *NtAN1b* was also analysed in leaves and roots of transgenic lines. Results showed that *StMYBA1* over-expression resulted in an induction of the endogenous bHLH TFs *NtAN1a* and *NtAN1b* and the expression of *NtAN1b* was higher than *NtAN1a* in both leaves and roots of all transgenic lines (Fig. 4).The expression profiles of the anthocyanin biosynthetic genes and bHLH genes are largely overlapping, suggesting that the expression of *NtAN1a* and *NtAN1b*, especially *NtANb1*, seems to be essential for anthocyanin production in transgenic tobacco leaves and roots. The tobacco MYB NtAN2, which is regulating anthocyanin production in tobacco (Pattanaik et al. 2010), was not induced by over-expression of *StMYBA1* (not detectable by qPCR; not shown).

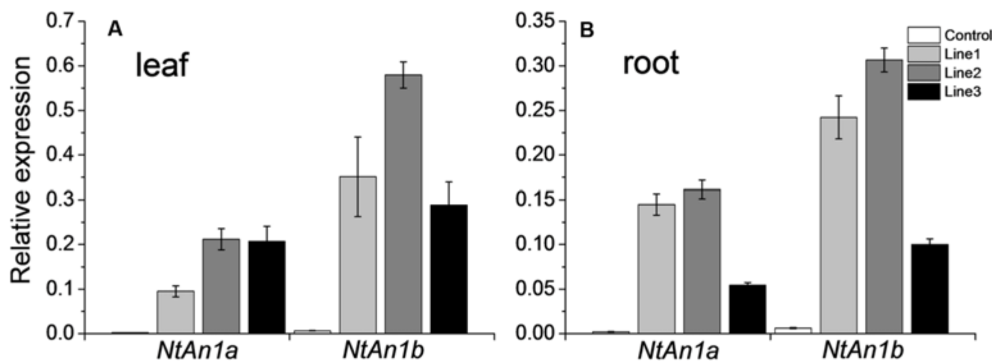

Fig. 4. Real-time qPCR analysis of expression levels of *NtAN1a* and *NtAN1b* in leaves (A) and roots (B) of transgenic tobacco plants overexpressing *StMYBA1.* The data represent the means ± SE of four technical replicates

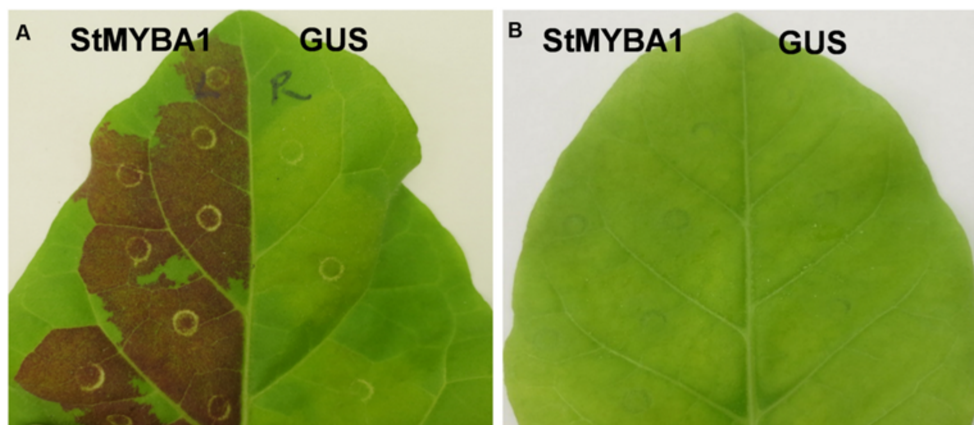

Fig. 5. Transient activation of anthocyanin biosynthesis regulated by StMYBA1 under natural light (A) and dark incubation (B). (A) Red coloration in tobacco leaves induced by the infiltration of tobacco leaves with StMYBA1 and GUS (as a control) under natural light; (B) in darkness

Anthocyanin Biosynthesis in Tobacco Regulated by StMYBA1 was Affected by Light

Environmental factors such as light, drought and temperature can strongly affect anthocyanin biosynthesis (Cominelli et al. 2008; Albert et al. 2009). Therefore, in this work, whether the anthocyanin biosynthesis of transgenic tobacco plants regulated by *StMYBA1* can be affected by light was further investigated. The phenotype and expression levels of several anthocyanin-related genes in tobacco leaves infiltrated with StMYBA1 were evaluated after 3d-incubation in darkness. The result showed that tobacco leaves infiltrated with StMYBA1 grown under natural light conditions accumulated intense red pigment, while tobacco plants grown in darkness conditions remained green foliage after three days (Fig. 5), suggesting that light is involved in the modulation of StMYBA1 activity in tobacco leaves. The real-time qPCR analysis showed that the anthocyanin biosynthetic genes including *NtCHS*, *NtCHI*, *NtF3'H*, *NtF3H*, *NtANS, NtDFR* and *NtUFGT* were down-regulated under dark incubation, in particular, the expression levels of two anthocyanin pathway genes – *NtDFR* and *NtANS* were reduced significantly in darkness (Fig. 6A).

As expected, expression of *StMYBA1* in *35S: StMYBA1* leaves was up-regulated in comparison with *35S: GUS* control plants, and there is no expression difference of *StMYBA1* between leaves grown under natural light and dark conditions (Fig. 6A).

Another crucial TF involved in anthocyanin biosynthesis is the bHLH protein. The expression of bHLH was also examined with qPCR (Fig. 6B). The expression of bHLH transcription factors *NtAN1a* and *NtAN1b* was significantly reduced in darkness compared with that under natural light (Fig. 6B), and expression profiles of the *NtAN1a* and *NtAN1b* genes and the two biosynthetic genes - *NtDFR* and *NtANS* are largely overlapping, suggesting that bHLHs are essential in regulating anthocyanin biosynthesis.

These results showed that light positively affected the anthocyanin biosynthesis and clearly suggested that there are other light-regulated factors involved in the regulation of anthocyanin biosynthesis, and is worthy of further investigation.

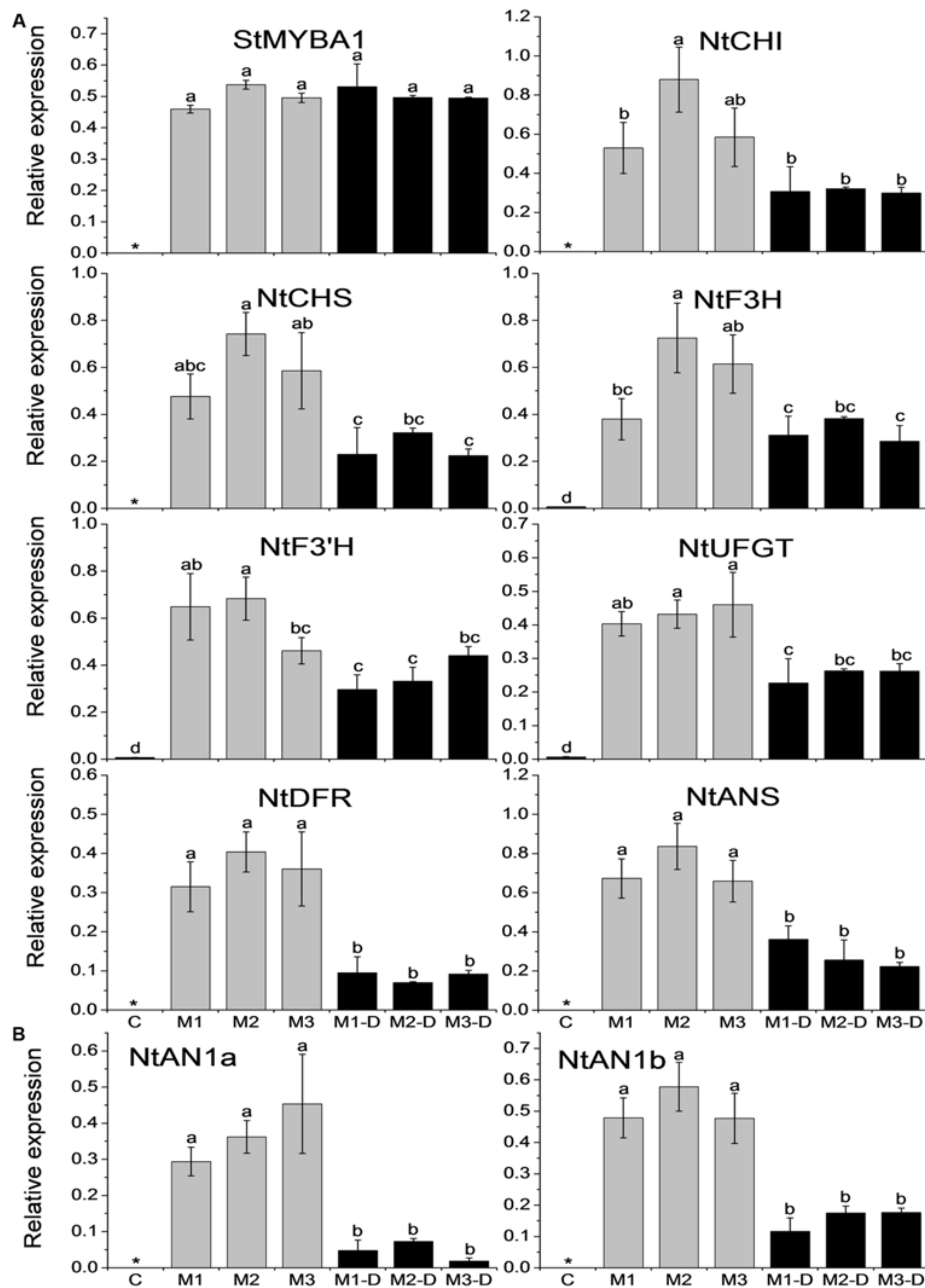

Fig. 6. Expression analysis of anthocyanin biosynthetic genes and transcription factors in leaves of tobacco plants infiltrated with GUS (control) and StMYBA1 under natural light and in darkness. (A) expression of *StMYBA1* and anthocyanin pathway genes, (B) bHLH TFs – *AN1a* and *AN1b* involved in anthocyanin biosynthesis. Error bars are the SE of three biological replicates. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance; bars with different letters (a, b, c, etc) indicate significant differences between means (least significant differences-LSD, $P < 0.05$). Genotypes denoted by *showed that no expression was detected. C represents control tobacco plants infiltrated with GUS. M1 to M3 and M1-D to M3-D represent tobacco plants, infiltrated with StMYBA1, were grown under natural light and in darkness, respectively

StMYBA1 Upstream Promoter Region Sequence Analysis

To investigate the *cis*-elements of *StMYBA1* promoter that could be related to light and other factors such as temperature and hormones, the 1.998-kb upstream promoter region from the start codon of *StMYBA1* was isolated and predicted in PLACE online database (Genbank accession No. KU991152). The putative *cis*-regulatory elements involved in light responsiveness and other environmental factors were identified in the *StMYBA1* promoter as shown in Table 1.

Table 1. Identification of putative *cis*-regulatory elements in the *StMYBA1* promoter.

Category	*cis*-regulatory element	Motif	Numbers of element
Light response	ACE	AAAACGTTTA	1
	ACE	ACGTGGA	1
	Box I	TTTCAAA	3
	Box 4	ATTAAT	5
	G-Box	CACGTT	1
	G-Box	CACGTC	1
	G-Box	CACGTA	1
Defense & stress response	TC-rich repeats	RTTTTCTTMM	3
Abscisic acid response	ABRE	TACGTG	1
Ethylene response	ERE	ATTTCAAA	2
Heat stress response	HSE	AAAAAATTTC	1
MeJA response	CGTCA-motif	CGTCA	1
	TGACG-motif	TGACG	1
Circadian control	Circadian	CAANNNNATC	6

The result showed that numerous *cis*-regulatory elements were identified, in which the light-responsive elements (LRE) such as Box I, G-Box, ACE, Box 4 and G-Box were the most abundant motifs. Three TC-repeats are defense and stress responsive. One ABRE motif (TACGTG) is abscisic acid (ABA)-responsive element. The other *cis*-regulatory elements including one ERE (ATTTCAAA) element involved in the ethylene response, one HSE (AAAAAATTTC) motif involved in the heat stress response, two MeJA-responsive *cis*-elements - CGTCA and TGACG and six circadian elements involved in circadian control.

Discussion

StAN1, coding for a R2R3 MYB, was shown to control potato skin colour and StMYBA1 was suggested as a likely pseudogene of StAN1 (Jung et al. 2009). Here, we further investigated the regulation mechanism of anthocyanin biosynthesis in transgenic tobacco overexpressing potato *StMYBA1* based on the previous transient assay result (Liu et al. 2016). When *StMYBA1* was stably transformed into tobacco plants, noticeable red pigment was accumulated in the leaves, flowers, ovaries and roots of transgenic plants (Fig. 1). In all three transgenic lines, especially line 2, large amounts of anthocyanins were detected (Fig. 2B). These results suggest that *StMYBA1* can regulate anthocyanin biosynthesis in potato.

The bHLH TFs interact with R2R3 MYB partners to form transcriptional complexes to regulate the anthocyanin biosynthetic genes. For example, in maize (*Zea mays*), the promoter of *DFR* is activated by the transcriptional complex of R2R3 MYB factor C1 and the bHLH TF B or R (Sainz et al. 1997). Exogenous MYB TFs can also affect endogenous bHLH TFs, for example, in petunia, the MYB TF *PhAn2* up-regulates expression of the bHLH TF *PhAn1* (Spelt et al. 2000). In *MrMYB1*-overexpressing transgenic tobacco plants, The expression of bHLH TFs *NtAN1a* and *NtAN1b* of petals and ovaries was highly induced by *MrMYB1*, however, the expression of bHLH TFs is extremely low in transgenic tobacco leaves, thus no anthocyanin was accumulated (Huang et al. 2013). In *LcMYB1* over-expressing tobacco plants, the upregulation of *NtAN1b* was noticeable in pigmented pedicel and leaf (Lai et al. 2014). In present work, whether the endogenous MYB and bHLH TFs in transgenic tobacco plants can by stimulated by overexpression of *StMYBA1* was investigated. The result showed that higher expression of endogenous *NtAN1a* and *NtAN1b*, especially *NtAN1b* in leaves and roots was detected, resulting in the high expression of late anthocyanin biosynthetic genes such as *NtANS* in leaves and *NtDFR* in roots. These results suggest that the expression of *NtAN1b* is essential for the anthocyanin accumulation in tobacco leaves and roots (Fig. 3; Fig. 4).

Light stimulates the expression of MYB TFs involved in anthocyanin biosynthesis, resulting in the red pigmentation in plants (Allan et al. 2008; Rowan et al. 2009; Li et al. 2012). The modulatory effect of light on anthocyanin accumulation in transgenic plants overexpressing regulatory genes has already been studied (Ray et al. 2003; Albert et al. 2009). In this study, both anthocyanin biosynthetic genes and bHLH TFs involved in anthocyanin biosynthesis were down-regulated in tobacco leaves infiltrated with *StMYBA1* under dark incubation. It suggests that the involvement of the other light-regulated factors in modulation of anthocyanin biosynthesis in tobacco plants, along with ongoing over-expression of *StMYBA1*. In Arabidopsis, apart from MBW complex, the transcriptional factor ELONGATED HYPOCOTYL 5 (HY5) is involved in the anthocyanin biosynthesis and responded to light

(Shin et al. 2007; ller et al. 2011). In darkness, the HY5, PAP1 and PAP2 of the Arabidopsis seedling are ubiquitinated by the CONSTITUTIVE PHOTOMORPHOGENIC 1/SUPPRESSOR OF PHYTOCHROME A-105 (COP1/SPA) complex and then degraded by the 26S proteasome (Maier et al. 2013). In apple, a B-box protein, MdCOL11 regulated the expression of *MdMYBA* and MdCOL11 was a target of MdHY5, which suggest that MdCOL11 is involved in MdHY5-mediated signal transduction and regulates anthocyanin accumulation in apple peel (Bai et al. 2014). Thus, our results suggest that there are other light-regulated factors possibly involved in the regulation of anthocyanin biosynthesis induced by *StMYBA1* in tobacco plants under dark conditions, and will be further investigated.

Our results demonstrate that StMYBA1 positively regulates anthocyanin biosynthesis based on the analysis of phenotype and gene expression. Furthermore, our results suggest that light-dependent factors are involved in the activating anthocyanin biosynthetic genes and regulatory bHLH TFs in *StMYBA1*-overexpressing transgenic tobacco plants. The role of *StMYBA1* in potato will be further investigated in future work.

Materials and Methods

Plant Materials

Purple potato cultivar 'Hei Meiren' (abbreviated as HM; purple peel and purple flesh) was from Gansu Agricultural University, China. *Nicotiana tabacum* was used for transformation, which was grown in a greenhouse under natural lighting (16hrs light/8hrs dark).

RNA, DNA Isolation and First-strand cDNA Synthesis

Total RNA samples of young leaves and roots of tobacco were extracted by using the PureLink Plant RNA Reagent Kit (Invitrogen, Carlsbad, CA, USA1) followed the manufacturer's instructions. The RNA was quantified by using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and quality was measured on a 1% (w/v) agarose gel. To remove genomic DNA contamination, the cDNA was treated with RQ RNase-free DNase (Promega, Fitchburg, WI, USA) and first-strand cDNA were synthesized by using oligo(dT) (QuantiTect Reverse Transcription Kit; Qiagen, Valencia, CA, USA). Genomic DNA was isolated from tuber tissue using the CTAB method (Murray and Thompson 1980).

Electroporation of *StMYBA1* Construct and Promoter Analysis

The pSAK277 construct for 35S:StMYBA1-1 was as previously described (Liu et al. 2016). The Genbank accession number for *StMYBA1-1* is KP317177. Binary vector carrying the pSAK277 construct were electroporated into *Agrobacterium tumefaciens* strain GV3101 (MP90).

The promoter of potato *StMYBA1* (1.998 kb upstream of *StMYBA1*) from HM was amplified by polymerase chain reaction (PCR) with one pair of primers: StMYBA1-PF (5'- GGTAGGACTGTGTTGG-TGTGATTGATG-3') and StMYBA1-PR (5'-CCATGGTCCCAACGA-TGCACACATAGGA-3') and inserted into the *Nco*I and *Xho*I restriction sites of pGreenII 0800-LUC vector (Hellens et al. 2005). Conserved cis-element motifs in the promoter were subjected to analysis by using online PLACE software tools (http://www.dna.affrc.go.jp/PLACE/).

Tobacco Transformation

Tobacco transformation was carried out by using a leaf disc transformation method (Horsch et al. 1985). The regenerating transgenic shoots were obtained on selective medium containing 3% (w/v) sucrose, 0.7% (w/v) agar (Taconic, Germantown, NY, USA), 1.0 mg l^{-1} 6-benzylaminopurine (BAP), 1.0 mg l^{-1} 1-Naphthaleneacetic acid (NAA), 300 mg l^{-1} Timentin (ticarcillin disodium and clavulanate potassium) and 150 mg l^{-1} kanamycin. These transgenic shoots were transferred to the greenhouse under long-day lighting (16hrs light/8hrs dark) after 1 month culture on medium.

Real-time Quantitive PCR (qPCR) Analysis of Gene Expression

Transgenic tobacco plants were obtained by kanamycin selection and further analysed by real-time qPCR. Amplification and analysis of target genes were performed on the LightCycler 480 Real-Time PCR System (Roche, Foster City, CA, USA). All reactions were carried out using the LightCycler 480 SYBR Green I Master Mix (Roche). Reactions were performed in quadruple using 1 µl cDNA, 5 µl 2×Master Mix, 0.5 µM each primer and 3 µl nuclease-free water to a total volume of 10 µl. The qPCR cycling system was as follows: 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 5 s, and elongation at 72°C for 10 s. Amplification was then followed by melting curve analysis at 65-95°C. For each gene, the cDNA sample was serially ten-fold diluted to generate a standard curve and the qPCR efficiency was obtained by analysing standard curve and then imported into expression data analysis. Tobacco actin (GenBank accession No. EU938079) and elongation factor-1 (GenBank accession No. D63396) were used for template normalization to calculate relative abundance with the ΔC_T method. The data was analysed with the LigherCycler 480 software version 1.5, Error bars represent the means ±SE of four technical replicate qPCR reactions. One-way ANOVA was used to determine the statistical significance.

The relative expression levels of *StMYBA1*, endogenous *NtAN2, NtAN1a*, *NtAN1b*, and the anthocyanin biosynthetic genes of chalcone synthase (*NtCHS*), chalcone flavanone isomerase (*NtCHI*), flavonoid 3'-monooxygenase (*NtF3'H*), flavanone 3 beta-hydroxylase (*NtF3H*), dihydroflavonol 4-reductase (*NtDFR*), anthocyanidin synthase (*NtANS*), anthocyanidin 3-O-glucosyltransferase (*NtUFGT*) were measured by real-time qPCR. The details of primer information were listed in Liu et al. (2016).

HPLC Analysis of Transgenic Tobacco Plants

For the stably *StMYBA1*-overexpressing tobacco plants, three tobacco leaves and roots from each of three transgenic tobacco lines were taken and pooled together to determine total anthocyanin content by HPLC, and empty vector pSAK277 was a negative control. For dual luciferase assays, three patches of tobacco leaves under light and dark treatments were pooled together for HPLC measurements, respectively. GUS was used as a negative control and three biological replicates were performed. Samples of freeze-dried tobacco leaves (1g FW) were pulverized and extracted in 5 ml solvent (methanol with 0.1%HCl) at a room temperature for 3 h in the darkness, then centrifuged for 10 min at 3500 rpm. A 500 µl aliquot of 20-fold diluted supernatant (with 20% methanol) was measured by HPLC (McGhie et al. 2005).

Transient Assays of Gene Function

N. tabacum was grown in the greenhouse as described above were used for infiltration. Cultures of *Agrobacterium tumefaciens* strain GV3101 were incubated for the dual luciferase assays. The strains

containing StMYBA1 and GUS fused to the 35S promoter in the pSAK277 vector were mixed (500 μl) and infiltrated into the abaxial leaf surface as for the transient color assays. Three leaves of the same plant were performed for each infiltration. Three tobacco plants infiltrated with StMYBA1 as described above were grown in the greenhouse, the other three plants infiltrated with StMYBA1 were covered with foil for dark treatment.

Acknowledgements

This research program is financially supported by the National Natural Science Foundation of China (grant No. 31601356), the Fostering Foundation for the Excellent Ph.D. Dissertation of Gansu Agricultural University (2013), Agricultural Biotechnology Research and Application Development Project of Gansu Province (grant No. GNSW-2015-15), Scientific Research Foundation of the Higher Education Institutions of Gansu Province (grant No. 2016A-030) and International Science & Technology Cooperation Program of China (grant No. 2014DFG31570).

Author's Contributions

YL performed RNA, DNA isolation, first-strand cDNA synthesis, tobacco transformation, transient assays, real-time quantitive PCR analysis and wrote the manuscript; LW performed electroporation of construct, promoter analysis and HPLC analysis; BY and JW analyzed the data; JZ and DW conceived, designed the experiments and revised the manuscript. All the authors agreed on the contents of the paper and post no conflicting interest.

References

Albert NW, Lewis DH, Zhang H, Irving LJ, Jameson PE, Davies KM (2009) Light-induced vegetative anthocyanin pigmentation in Petunia. J Exp Bot:erp097

Allan AC, Hellens RP, Laing WA (2008) MYB transcription factors that colour our fruit. Trends Plant Sci 13:99–102

Bai S, Saito T, Honda C, Hatsuyama Y, Ito A, Moriguchi T (2014) An apple B-box protein, MdCOL11, is involved in UV-B-and temperature-induced anthocyanin biosynthesis. Planta 240:1051–1062

Bazzano LA, Serdula MK, Liu S (2003) Dietary intake of fruits and vegetables and risk of cardiovascular disease. Curr Atheroscler Rep 5:492–499

Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell 12:2383–2393

Borovsky Y, Oren-Shamir M, Ovadia R, De Jong W, Paran I (2004) The *A* locus that controls anthocyanin accumulation in pepper encodes a MYB transcription factor homologous to *Anthocyanin2* of *Petunia*. Theor Appl Genet 109:23–29

Chagné D, Lin-Wang K, Espley RV, Volz RK, How NM, Rouse S, Brendolise C, Carlisle CM, Kumar S, De Silva N (2013) An ancient duplication of apple MYB transcription factors is responsible for novel red fruit-flesh phenotypes. Plant Physiol 161:225–239

Cominelli E, Gusmaroli G, Allegra D, Galbiati M, Wade HK, Jenkins GI, Tonelli C (2008) Expression analysis of anthocyanin regulatory genes in response to different light qualities in *Arabidopsis thaliana*. J Plant Physiol 165:886–894

D'Amelia V, Aversano R, Batelli G, Caruso I, Castellano Moreno M, Castro-Sanz AB, Chiaiese P, Fasano C, Palomba F, Carputo D (2014) High AN1 variability and interaction with basic helix-loop-helix co-factors related to anthocyanin biosynthesis in potato leaves. Plant J 80:527–540

Dubos C, Le Gourrierec J, Baudry A, Huep G, Lanet E, Debeaujon I, Routaboul JM, Alboresi A, Weisshaar B, Lepiniec L (2008) MYBL2 is a new regulator of flavonoid biosynthesis in *Arabidopsis thaliana*. Plant J 55:940–953

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 RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J 49:414–427

Feller A, Machemer K, Braun EL, Grotewold E (2011) Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. Plant J 66:94–116

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. Plant J 53:814–827

Grotewold E (2006) The genetics and biochemistry of floral pigments. Annual Review of Plant Biology 57:761–780

Hellens RP, Allan AC, Friel EN, Bolitho K, Grafton K, Templeton MD, Karunairetnam S, Gleave AP, Laing WA (2005) Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Plant Methods 1:13

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7:1071

Horsch R, Fry J, Hoffmann N, Eichholtz D, Rogers S, Fraley R (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Huang Y-J, Song S, Allan AC, Liu X-F, Yin X-R, Xu C-J, Chen K-S (2013) Differential activation of anthocyanin biosynthesis in *Arabidopsis* and tobacco over-expressing an R2R3 MYB from Chinese bayberry. Plant Cell Tiss Org Cult 113:491–499

Islam MS, Jalaluddin M, Garner JO, Yoshimoto M, Yamakawa O (2005) Artificial shading and temperature influence on anthocyanin compositions in sweetpotato leaves. HortScience 40:176–180

Jung CS, Griffiths HM, De Jong DM, Cheng S, Bodis M, Kim TS, De Jong WS (2009) The potato *developer* (*D*) locus encodes an R2R3 MYB transcription factor that regulates expression of multiple anthocyanin structural genes in tuber skin. Theor Appl Genet 120:45–57

Kim S-H, Lee J-R, Hong S-T, Yoo Y-K, An G, Kim S-R (2003) Molecular cloning and analysis of anthocyanin biosynthesis genes preferentially expressed in apple skin. Plant Sci 165:403–413

Kobayashi S, Goto-Yamamoto N, Hirochika H (2004) Retrotransposon-induced mutations in grape skin color. Science 304:982–982

Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci 10:236–242

Lai B, Li X-J, Hu B, Qin Y-H, Huang X-M, Wang H-C, Hu G-B (2014) LcMYB1 is a key determinant of differential anthocyanin accumulation among genotypes, tissues, developmental phases and ABA and light stimuli in *Litchi chinensis*. PLoS ONE 9:e86293

Li Y-Y, Mao K, Zhao C, Zhao X-Y, Zhang H-L, Shu H-R, Hao Y-J (2012) MdCOP1 ubiquitin E3 ligases interact with MdMYB1 to regulate light-induced anthocyanin biosynthesis and red fruit coloration in apple. Plant Physiol 160:1011–1022

Liu Y, Lin-Wang K, Espley RV, Wang L, Yang H, Yu B, Dare A, Varkonyi-Gasic E, Wang J, Zhang J, Wang D, Allan AC (2016) Functional diversification of the potato R2R3 MYB anthocyanin activators AN1, MYBA1, and MYB113 and their interaction with basic helix-loop-helix cofactors. J Exp Bot:erw014

Maier A, Hoecker U (2015) COP1/SPA ubiquitin ligase complexes repress anthocyanin accumulation under low light and high light conditions. Plant signaling & behavior 10:e970440

Maier A, Schrader A, Kokkelink L, Falke C, Welter B, Iniesto E, Rubio V, Uhrig JF, HHülskamp M, Hoecker U (2013) Light and the E3 ubiquitin ligase COP1/SPA control the protein stability of the MYB transcription factors PAP1 and PAP2 involved in anthocyanin accumulation in Arabidopsis. Plant J 74:638–651

Martens S, Preuß A, Matern U (2010) Multifunctional flavonoid dioxygenases: Flavonol and anthocyanin biosynthesis in *Arabidopsis thaliana* L. Phytochemistry 71:1040–1049

Mathews H, Clendennen SK, Caldwell CG, Liu XL, Connors K, Matheis N, Schuster DK, Menasco D, Wagoner W, Lightner J (2003) Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell 15:1689–1703

McGhie TK, Hunt M, Barnett LE (2005) Cultivar and growing region determine the antioxidant polyphenolic concentration and composition of apples grown in New Zealand. J Agric Food Chem 53:3065–3070

Murray M, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4326

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

Payyavula RS, Singh RK, Navarre DA (2013) Transcription factors, sucrose, and sucrose metabolic genes interact to regulate potato phenylpropanoid metabolism. J Exp Bot 64:5115–5131

Paz-Ares J, Ghosal D, Wienand U, Peterson P, Saedler H (1987) The regulatory *c1* locus of *Zea mays* encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. The EMBO Journal 6:3553

Ray H, Yu M, Auser P, Blahut-Beatty L, McKersie B, Bowley S, Westcott N, Coulman B, Lloyd A, Gruber MY (2003) Expression of anthocyanins and proanthocyanidins after transformation of alfalfa with maize *Lc*. Plant Physiol 132:1448–1463

Rosinski JA, Atchley WR (1998) Molecular evolution of the Myb family of transcription factors: evidence for polyphyletic origin. J Mol Evol 46:74–83

Rowan DD, Cao M, Linâ Wang K, Cooney JM, Jensen DJ, Austin PT, Hunt MB, Norling C, Hellens RP, Schaffer RJ (2009) Environmental regulation of leaf colour in red 35S: PAP1 *Arabidopsis thaliana*. New Phytol 182:102–115

Sainz MB, Grotewold E, Chandler VL (1997) Evidence for direct activation of an anthocyanin promoter by the maize C1 protein and comparison of DNA binding by related Myb domain proteins. Plant Cell 9:611–625

Shin J, Park E, Choi G (2007) PIF3 regulates anthocyanin biosynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in Arabidopsis. Plant J 49:981–994

Spelt C, Quattrocchio F, Mol JN, Koes R (2000) anthocyanin1 of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. Plant Cell 12:1619–1631

Wang L, Lee IM, Zhang SM, Blumberg JB, Buring JE, Sesso HD (2009) Dietary intake of selected flavonols, flavones, and flavonoid-rich foods and risk of cancer in middle-aged and older women. Am J Clin Nutr 89:905–912

Zhang Y, Jung CS, De Jong WS (2009) Genetic analysis of pigmented tuber flesh in potato. Theor Appl Genet 119:143–150