DOI: 10.1007/s10535-015-0548-4 BIOLOGIA PLANTARUM 59 (4): 677-685, 2015

RNAi-mediated silencing and overexpression of the *FaMYB1* gene and its effect on anthocyanin accumulation in strawberry fruit

Y. KADOMURA-ISHIKAWA[1,2], K. MIYAWAKI[2]*, A. TAKAHASHI[1], and S. NOJI[2]

Department of Nutrition, Faculty of Medicine, University of Tokushima, Tokushima 770-8503, Japan[1]
Department of Life System, Institute of Technology and Science, University of Tokushima,
Tokushima 770-8506, Japan[2]

Abstract

Strawberry (*Fragaria* × *ananassa*) contains anthocyanins which are important secondary metabolites and key contributors to the antioxidant capacity and nutritional value of the fruit. Anthocyanin biosynthetic genes have been identified. However, the detailed mechanism responsible for anthocyanin accumulation and regulation of biosynthetic genes during strawberry fruit ripening remain unclear. In the present study, we examined the effect of a *Fragaria* × *ananassa myeloblastosis 1* homolog, *FaMYB1*, on anthocyanin accumulation in the strawberry fruit receptacle. Expression analysis shows that *FaMYB1* transcripts increased in response to irradiance but not to abscisic acid treatments. Down-regulation of *FaMYB1* was achieved *in planta* using *Agrobacterium*-mediated RNA interference (RNAi). As a result, *FaMYB1*-RNAi fruits exhibited a significant increase in anthocyanin content. Conversely, overexpression of *FaMYB1* resulted in a decrease in anthocyanin content. Overexpression of *FaMYB1* also significantly reduced expression of genes encoding anthocyanidin synthase and flavonoid glycosyltransferase, whereas down-regulation of *FaMYB1* resulted in a significant decrease in the amount of transcripts of leucoanthocyanidin reductase. These data suggest that FaMYB1 might negatively control anthocyanin biosynthesis in the strawberry fruit at the branching-point of anthocyanin/proanthocyanidin biosynthesis.

Additional key words: abscisic acid, flavonoid pathway, MYB transcription factor, *Fragaria* × *ananassa.*

Introduction

Fruit ripening is a genetically programmed process accompanied by notable changes in fruit flesh, including colour, flavour, texture, and aroma. According to ripening, fruits have been divided into the following two groups: climacteric fruits, such as apple and tomato, and non-climacteric fruits, such as grape and strawberry (Osorio *et al.* 2013). A peak in respiration and a concomitant burst in ethylene accompany climacteric fruit ripening, whereas in a non-climacteric fruit, ethylene production remains at a very low level (Alexander and Grierson 2002). Tomato has been used as climacteric fruit model in several studies to investigate the molecular mechanisms of ethylene action, and results suggest the ethylene burst characterizing a climacteric fruit is required for normal fruit ripening (Klee and Giovannoni 2011). In contrast, the detailed ripening mechanisms of a non-climacteric fruit, including strawberry, remain largely unknown.

Anthocyanins are synthesized *via* the flavonoid biosynthesis pathway (Fig. 1). Genes that encode the enzymes directly mediating anthocyanin and other flavonoid formation are highly regulated (Cominelli *et al.*

Submitted 11 September 2014, *last revision* 6 April 2015, *accepted* 15 April 2015.

Abbreviations: ABA - abscisic acid; ANR - anthocyanidin reductase; ANS - anthocyanidin synthase; CHI - chalcone isomerase; CHS - chalcone synthase; DFR - dihydroflavonol-4-reductase; FGT - flavonoid glycosyltransferase; F3H - flavanone-3-hydroxylase; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; LAR - leucoanthocyanidin reductase; MYB - myeloblastosis; PA - proanthocyanidin; TF - transcription factor.

Acknowledgements: We are grateful to Koichi Hayashi for donating strawberry fruit samples. We also thank Toshifumi Miki and Keisuke Hirota (the Tokushima Agricultural Research Center, Japan) for invaluable advice and donation of strawberry fruit samples. This work was supported by the Ourgenic Co., Tokushima, Japan, and by the LED-Life project of the University of Tokushima, Japan.

* Corresponding author; fax: (+81) 88 6337092, e-mail: miyawaki.katsuyuki.1@tokushima-u.ac.jp

2008, Miyawaki *et al.* 2012) (Fig. 1). During strawberry fruit ripening, gene transcriptions and activities of chalcone synthase (FaCHS), chalcone isomerase (FaCHI), flavanone-3-hydroxylase (FaF3H), dihydroflavonol-4-reductase (FaDFR), anthocyanidin synthase (FaANS), and flavonoid glycosyltransferase (FaFGT) are higher at later fruit developmental stages, thereby resulting in anthocyanin accumulation at ripening (Almeida *et al.* 2007, Carbone *et al.* 2009). This pathway is also regulated in response to different developmental and environmental cues (Winkel-Shirley 2001, Vogt 2010).

A complex of MYB transcription factors (TFs), basic helix-loop-helix (bHLH) TFs, and WD-repeat proteins regulate flavonoid pathway gene expression (Baudry *et al.* 2004). The complex was designated as MYB-bHLH-WD40 (MBW). Koes *et al.* (2005) proposed a model to characterize activation of flavonoid pathway genes, including interactions among regulators, leading to formation of transcriptional complexes which subsequently recognize structural gene promoters. The R2R3 MYBs are well-known regulators of anthocyanin biosynthesis, and several R2R3 MYBs function as repressors, such as the *Fragaria* × *ananassa* FaMYB1 (Aharoni *et al.* 2001, Paolocci *et al.* 2011), *F. chiloensis* FcMYB1 (Salvatierra *et al.* 2013), *Arabidopsis* AtMYB4 (Jin *et al.* 2000), and *Antirrhinum* AmMYB308 (Tamagnone *et al.* 1998). Aharoni *et al.* (2001) reported that *FaMYB1* overexpression in tobacco plants is associated with a decreased flower anthocyanin content. Paolocci *et al.* (2011) also reported that *FaMYB1* overexpression in *Lotus corniculatus* inhibits biosynthesis of proanthocyanidins (PAs). Recently, Salvatierra *et al.* (2013) has showed that down-regulation of the *FcMYB1,* an ortholog of the *FaMYB1* isolated from native white *F. chiloensis* subsp. *chiloensis* fruits, increases anthocyanin content. Medina-Puche *et al.* (2014) has reported that down-regulation of the *FaMYB1* in strawberry fruits exhibits no-effect on fruit coloration. However, evidence obtained from analyses of these heterologous systems is limited, and variable effects on fruit coloration were observed between FaMYB1 and its orthologs in *Fragaria* species. Therefore, FaMYB1 function in *Fragaria* species, including strawberry, remains still elusive.

Anthocyanin biosynthesis in plants is highly regulated by irradiance (Cominelli *et al.* 2008, Kami *et al*. 2010). In strawberry, blue radiation is most effective in anthocyanin biosynthesis induction. In this process, strawberry phototropin 2 (FaPHOT2), which is one of the photoreceptors, plays a role in blue radiation perception, at least in part (Miyawaki *et al.* 2012, Kadomura-Ishikawa *et al.* 2013). Recently, it has been reported that anthocyanin biosynthesis is also regulated by abscisic acid (ABA). Knockdown of an ABA receptor or an ABA synthetic enzyme leads to an uncoloured phenotype (Chai *et al.* 2011, Jia *et al.* 2011). In addition, type 2C protein phosphatase ABI1 involved in ABA signaling, negatively regulates strawberry fruit ripening (Jia *et al.* 2013). However, signal transduction following irradiance or ABA treatment remains to be characterized.

In the present study, we investigated *FaMYB1* transcription during strawberry fruit ripening and its expression in response to irradiance and ABA. We also analyzed the effects of *FaMYB1* overexpression or down-regulation on strawberry fruit coloration to elucidate FaMYB1 function in anthocyanin biosynthesis in the strawberry fruit.

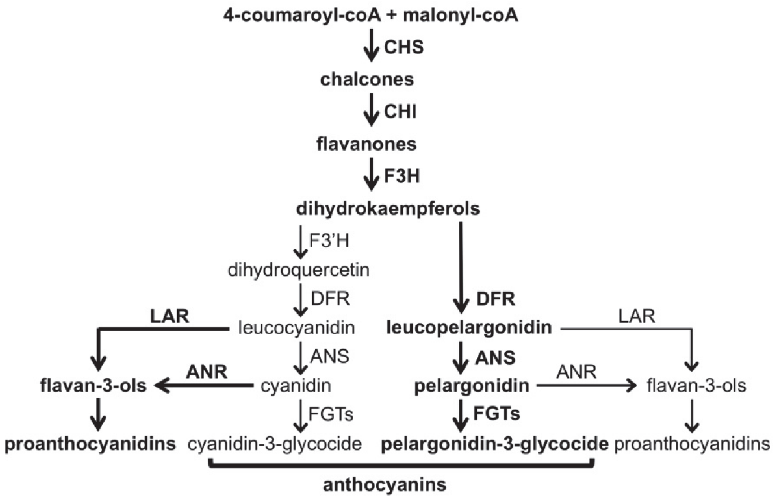

Fig. 1. Flavonoid biosynthesis in strawberry. Enzymes for each step are shown in *bold* and include: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), dihydroflavonol-4-reductase (DFR), anthocyanidin synthase (ANS), flavonoid glycosyltransferases (FGTs), anthocyanidin reductase (ANR), and leucoanthocyanidin reductase (LAR). *Distinctive arrows* indicate the major pathways.

Materials and methods

Plants and treatments: Octaploid strawberry plants (*Fragaria* × *ananassa* Duch. cv. Sachinoka) were cultivated in a greenhouse from winter to spring. Strawberry fruits were harvested at each of the six developmental stages: small green (SG), medium green (MG), big green (BG), white (Wh), turning (Tu), and red (Re) (Fait *et al.* 2008). Harvested fruits were subsequently sterilized according to Miyawaki *et al.* (2012), then placed on plates and maintained at 23 °C.

We previously demonstrated that anthocyanin biosynthesis is induced by irradiance (Kadomura-Ishikawa *et al.* 2013). Therefore, the effect of irradiance on *FaMYB1* transcription was evaluated in strawberry fruits using a quantitative real-time reverse transcription-PCR (qRT-PCR). Harvested Wh stage strawberry fruits were placed in a growth chamber (*MLR-351*, *SANYO*, Osaka, Japan) at a temperature of 23 °C, a 16-h photoperiod, and an irradiance of 110 μmol m^{-2} s^{-1} provided by fluorescent tubes (*FL40SS W*, *Panasonic*, Osaka, Japan) for 6 d, whereas control plants were maintained in the dark.

Abscisic acid was shown to be a key regulator of anthocyanin accumulation in strawberry fruits (Chai *et al.* 2011, Jia *et al.* 2011, 2013). Therefore, harvested strawberry fruits (the Wh stage) were also treated with ABA (*Sigma*, Osaka, Japan) or an ABA synthesis inhibitor fluridone (*Fluka*, Osaka, Japan) according to Chai *et al.* (2011) with the following modifications. A solution of 50 μM ABA or 500 μM fluridone (0.2 cm^3) was injected into harvested strawberry fruits on days 0, 2, and 4 using a 1-cm^3 syringe. Distilled water was used as control. The treated fruits were then placed in the growth chamber and maintained at 23 °C in the dark for 7 d.

Extraction and determination of anthocyanin content: A total anthocyanin content was measured using a spectrophotometer *OPTIMA SP-300* (Tokyo, Japan) according to Kortstee *et al.* (2011). Achenes were removed and subsequently each receptacle was chopped into 1 - 2 g pieces. These pieces were soaked in 5 - 10 volumes of methanol containing 1 % (m/v) hydrochloric acid and left overnight. The samples were centrifuged at 3 800 *g* for 10 min, then the supernatants were collected and transferred to fresh tubes. After a second centrifugation at 20 400 *g* for 5 min, the supernatants were again transferred to fresh tubes, then absorbance values were measured at 530 and 657 nm to correct for chlorophyll content. Student's *t*-test was used to compare the samples after the ABA or fluridone treatment against controls. The statistical analysis consisted of a variance analysis (*ANOVA*) with post-hoc Dunnet's test to determine significant differences between the means of anthocyanin accumulation of the samples in experiments of down-regulation and overexpression of the *FaMYB1*.

RNA extraction and cDNA synthesis: Prior to RNA isolation, achenes were removed from receptacles. Total RNA was subsequently isolated using an RNA kit with *Plant RNA Isolation Aid* (*Ambion*, Carlsbad, CA, USA). Total RNA obtained was treated with DNase (*TURBO DNA-free*™ kit, *Ambion*) following the manufacturer's instructions to remove genomic DNA contamination. The RNA samples were considered DNA free when no amplicons corresponding to analyzed genes were observed, using RNA as template under the qRT-PCR conditions. First-strand cDNA was synthesized from total RNA (1 μg) using a *Superscript III First-Strand* synthesis system (*Invitrogen*, Tokyo, Japan) with oligo (dT)20 primers attached to the system according to the manufacturer's instructions.

Analysis by qRT-PCR was performed using an *ABI 7900HT* system (*Applied Biosystems*, Tokyo, Japan) with gene specific primers (GSPs). In strawberry, two *DFR* genes exist (GenBank No. AY695812 and strawberry gene ID: GENE29482, not deposited in GenBank). We named AY695812 and GENE29482 as *FaDFR1* and *FaDFR2*, respectively. The *FaMYB1* gene primer sequences were designed based on GenBank data [GenBank No. AF401220; Aharoni *et al.* (2001)]. Sequences for flavonoid pathway genes except *FaDFR2* were obtained from previous reports (Hoffmann *et al.* 2006, Almeida *et al.* 2007, Salvatierra *et al.* 2010, Miyawaki *et al.* 2012). Primer sequences for the *FaDFR2* gene were designed based on GENE29482. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (GenBank No. AB363963) was used as internal control. All primer sequences are listed in Table 1 Suppl. The PCR reaction mixtures included 1 ng of cDNA, 200 nM each primer, and 5 mm^3 of 2× *Power SYBR Green PCR Master Mix* (Applied Biosystems). After incubation of the reaction mixtures at 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min were performed. Target quantity in each sample was normalized to a reference gene according to Salvatierra *et al.* (2010) using the comparative ($2^{-\Delta\Delta Ct}$) method based on the manufacturer's recommendations. To determine the efficiency of each particular qRT-PCR, melting curves were also analyzed to ensure the existence of a single amplification peak corresponding to a unique molecular species. Furthermore, we confirmed a single product in each PCR product by gel electrophoresis. *ANOVA* with post-hoc Dunnet's test was applied to determine significant differences between the relative expression means of samples, and Student's *t* test was used to compare the irradiated samples against their corresponding dark controls.

Plasmid construction for RNA interference (RNAi) and overexpression assays: Plasmid construction for

RNAi experiments was performed according to Kadomura-Ishikawa *et al.* (2013) with the exception of *FaMYB1* gene fragments isolation. The *FaMYB1* fragments were amplified using sense (S) and anti-sense (AS) primers (Table 2 Suppl.) and cDNA from receptacles. The amplified products were ligated into a pRI-intron vector derived from pRI 201-AN (*Takara*, Shiga, Japan), a plasmid that contains an intron of the strawberry quinone oxidoreductase gene (GenBank No. AY158836). The resulting construct was named pRI-*myb1-ihpRNAi* (pRI-myb1i).

For overexpression assays, the *FaMYB1* open reading frame (ORF) was amplified from cDNA of receptacles using PCR primers (Table 2 Suppl.). The PCR products were cloned into a *pGEM-T Easy* vector system (*Promega*, Madison, WI, USA). The *FaMYB1* ORF was cleaved from the pGEM-T Easy vector and ligated into the pRI-201-AN vector. The resulting construct was named pRI-MYB1-overexpression (pRI-*MYB1-OE*). The sequence of each construct was confirmed using a *BigDye Terminator v. 1.1* cycle sequencing kit and an *ABI PRISM 310* genetic analyzer (*Applied Biosystems*).

***Agrobacterium* infiltration of plasmids:** The pRI-intron, pRI-*myb1i*, and pRI-*MYB1-OE* constructs were introduced into *Agrobacterium tumefaciens* L. strain GV2260, and transformation of strawberry fruits was achieved using an *Agrobacterium*-infiltration method according to Hoffmann *et al.* (2006) and Miyawaki *et al.* (2012). Suspensions of *Agrobacterium* harboring each construct were injected into strawberry fruit on days 0, 2, and 4, and they were maintained in the above mentioned growth chamber for 6 d. The pRI-intron-infiltrated fruits were used as experimental controls.

Results

Expression of *FaMYB1* during fruit ripening in strawberry was analyzed by the qRT-PCR. During six fruit growth stages, the *FaMYB1* expression gradually decreased during the first four stages (SG to Wh) and then increased during the Tu and Re stages (Fig. 2).

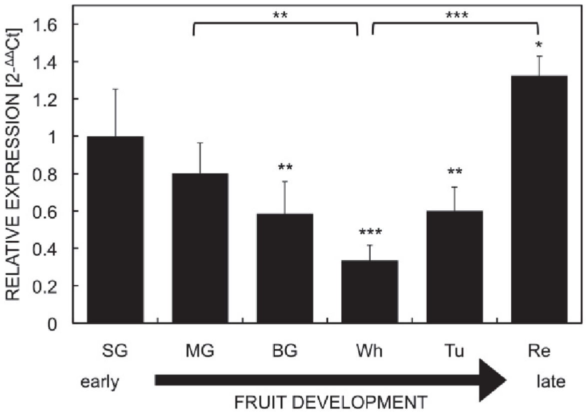

Fig. 2. The expression of *FaMYB1* during six developmental stages of strawberry fruits. Relative gene expression levels of the *FaMYB1* gene were normalized using *FaGAPDH*. These stages included: small green (SG), medium green (MG), big green (BG), white (Wh), turning (Tu), and red (Re). The normalized expression at the SG stage was set to 1. Data are expressed as means ± SD, $n = 4$. *Asterisks* indicate significant differences from the SG stage or the Wh stage (*brackets*) at * - $P < 0.05$, ** - $P < 0.01$, and *** - $P < 0.001$.

The effects of irradiance on *FaMYB1* expression was evaluated in strawberry fruits using the qRT-PCR. In the irradiated Wh fruits, the *FaMYB1* expression significantly increased at day 2 ($P < 0.01$), however, at days 4 and 6, it changed less ($P < 0.05$). In contrast, the *FaMYB1* expression in the controls under the dark remained unchanged (Fig. 3).

Further, the effects of exogenous ABA and the ABA synthetic inhibitor (fluridone) on anthocyanin accumulation and *FaMYB1* expression were analyzed. Specifically, we performed this experiment under dark conditions to exclude the effect of irradiance on anthocyanin accumulation in fruits. The anthocyanin content significantly increased ($P < 0.01$) after the ABA treatment and significantly decreased ($P < 0.001$) after the fluridone treatment compared to the control fruits (Fig. 4). In contrast, the qRT-PCR assays did not detect any significant changes in the *FaMYB1* expression induced by ABA or fluridone (Fig. 4).

To investigate a relationship between anthocyanin

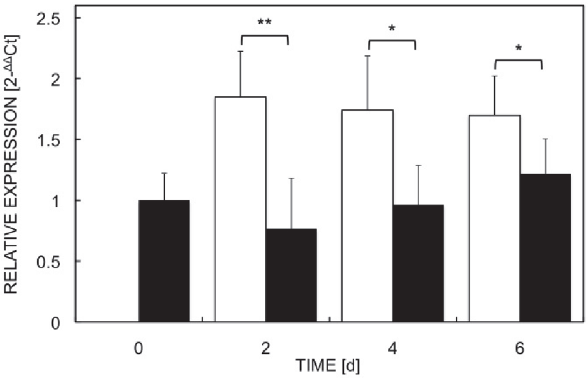

Fig. 3. The effect of irradiance of 100 µmol m^{-2} s^{-1} (a 16-h photoperiod) on *FaMYB1* expression in fruits harvested at white stage. Using the qRT-PCR, the expression of *FaMYB1* was normalized by the *FaGAPDH* expression, and the normalized expression at day 0 under the dark was set to 1. Means ± SD, $n = 4 - 5$. *Asterisks* indicate significant differences between irradiated fruits (*white bars*) and those maintained in the dark (*black bars*) (Student's *t*-test; * - $P < 0.05$, ** - $P < 0.01$).

accumulation and FaMYB1 in strawberry fruits, were performed *FaMYB1-RNAi* expression or *FaMYB1* gene overexpression in strawberry fruits *in planta*. The *FaMYB1* expression in the *FaMYB1-RNAi* fruits was 3.5-fold ($P < 0.05$) lower and in the*FaMYB1-OE* fruits were 83-fold higher ($P < 0.001$) than in the control fruits (Fig. 5). Moreover, colouring of the two treatment groups was characterized by an accumulation of anthocyanins in *FaMYB1-RNAi versus* a reduction in *FaMYB1-OE* compared to the control fruits (Fig. 5). Specifically, the total anthocyanin content of the *FaMYB1-RNAi* fruits increased by ~26 % and was 402.9 ± 21.6 µg g^{-1}(f.m.) and in the *FaMYB1-OE* fruits decreased by ~27 % and was 231.5 ± 20.7 µg g^{-1}(f.m.) as compared to the control fruits where it was 318.8 ± 24.8 µg g^{-1}(f.m.); these changes were significant at $P < 0.001$ (Fig. 5).

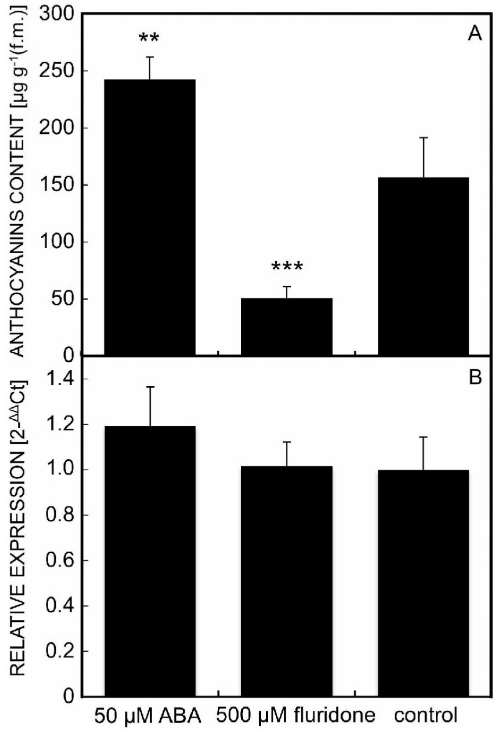

Fig. 4. The effect of ABA (50 µM) or an ABA synthetic inhibitor fluridone (500 µM) injected into harvested white stage fruits on anthocyanin accumulation (*A*) and *FaMYB1* expression (*B*). Control fruits were injected with distilled water. *Asterisks* indicate significant differences from controls (** - $P < 0.01$, *** - $P < 0.001$). The relative expression level of *FaMYB1* was normalized using *FaGAPDH*, and the normalized expression of the controls was set to 1. Data are expressed as means ± SD, $n = 4$ - 6.

To characterize FaMYB1 effects on anthocyanin accumulation at the molecular level, the expression of genes coding enzymes from the flavonoid pathway were determined in the *FaMYB1-RNAi* and *FaMYB1-OE* fruits using the qRT-PCR. In the *FaMYB1-RNAi* fruits, the *FaF3H* expression was significantly lower ($P < 0.05$), whereas the *FaCHS*, *FaCHI*, *FaDFR1, FaDFR2*, *FaANS*, and *FaFGT* expressions remained unchanged compared to the control fruits (Fig. 6). In contrast, the *FaDFR1* expression was significantly higher ($P < 0.01$), whereas the *FaANS* and *FaFGT* expressions were significantly lower ($P < 0.01$ in each case) in the *FaMYB1-OE* fruits compared to the controls (Fig. 6). Furthermore, significant differences were not detected in the *FaCHS*, *FaCHI*, *FaF3H*, and *FaDFR2* expressions in the *FaMYB1-OE* fruits and control fruits (Fig. 6).

Expression of *FaANR* and *FaLAR* genes, which encode PA synthetic enzymes (Fig. 1), was also determined in the *FaMYB1-RNAi* and *FaMYB1-OE* fruits. In the *FaMYB1-RNAi* fruits, the *FaLAR* expression was significantly lower ($P < 0.01$), whereas the *FaANR* expression showed no significant difference (Fig. 6) compared to the control fruits. In the *FaMYB1-OE* fruits, the *FaANR* expression was significantly lower ($P < 0.05$) and the *FaLAR* expression showed no significant difference compared to the control fruits (Fig. 6).

Discussion

Heterologous transformation experiments have demonstrated that FaMYB1 can regulate anthocyanin accumulation (Aharoni *et al.* 2001, Paolocci *et al.* 2011). In contrast to the *FaMYB1* heterologous transformation experiments, down-regulation of *FaMYB1* in strawberry fruits has no effect on fruit coloration (Medina-Puche *et al.* 2014). However, the effects of *FaMYB1* over-expression on strawberry fruits are still unclear. Therefore, detailed FaMYB1 function in strawberry remains elusive. In the present study, we focused on characterizing the role of FaMYB1 in strawberry fruits. The expression analysis of *FaMYB1* during fruit ripening using cv. Sachinoka revealed a two phase expression pattern; a decrease during the first four stages of fruit ripening followed by an increase during the last two stages. These data are consistent with results reported by Aharoni *et al.* (2001) for *FaMYB1* expression patterns in cv. Elsanta based on Northern blot analysis. Almeida *et al.* (2007), using the qRT-PCR and the *actin* gene as internal control, reported that *FaMYB1* expression in cv. Queen Elisa continued to increase during fruit ripening with the highest expression at the Re stage. We also analyzed *FaMYB1* expression using the *actin* gene as internal control and obtained a two phase expression pattern (results not shown) like in experiments when we used *GADPH* as reference gene. It is possible that cultivar differences between studies (*i.e.*, the present study and Almeida *et al.* 2007) may be responsible for lack of congruency in the results.

In many fruits, such as apple, peach, pear, grape, bilberry, cranberry, litchi, and strawberry, expression of flavonoid pathway genes as well as anthocyanin accumulation are induced by irradiance (Dussi *et al.* 1995, Kim *et al.* 2003, Kataoka and Beppu 2004, Zhou and Singh 2004, Jia *et al.* 2005, Anttonen *et al.* 2006, Carbone *et al.* 2009, Josuttis *et al.* 2010, Wei *et al.* 2011, Azuma *et al.* 2012, Miyawaki *et al.* 2012, Uleberg *et al.* 2012, Kadomura-Ishikawa *et al.* 2013). However, for non-climacteric fruits, such as strawberry, ABA is also one key regulator of anthocyanin biosynthesis (Chai *et al.* 2011, Jia *et al.* 2011, 2013). In the present study, however, *FaMYB1* was regulated by radiation and not by ABA or fluridone although the ABA and fluridone treatments were associated with a significant increase and decrease in anthocyanin content, respectively. We also investigated *FaANS* and *FaFGT* which were directly involved in anthocyanin biosynthesis (Fig. 1). The expression patterns of both genes were consistent with anthocyanin content (data not shown) indicating that the anthocyanin content and anthocyanin biosynthetic genes expression were affected by radiation and ABA.

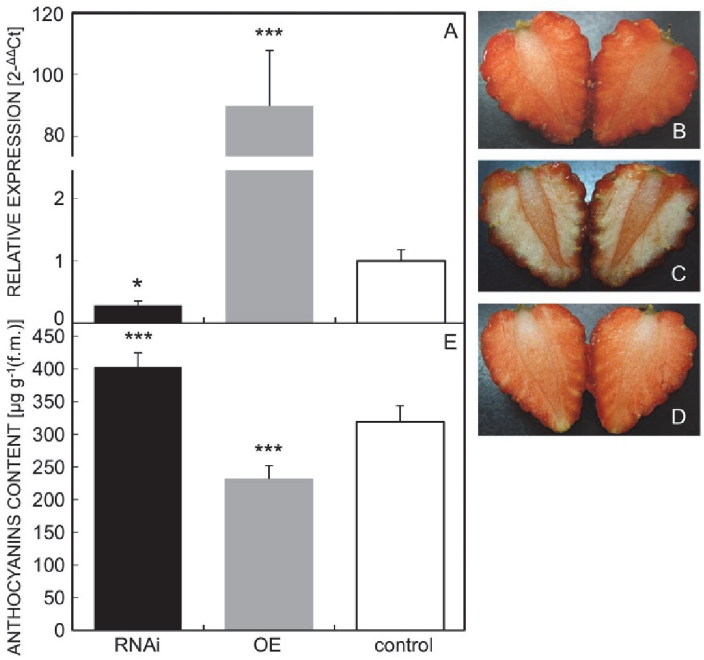

Fig. 5. The expression of *FaMYB1* and anthocyanin content in *FaMYB1-RNAi* and *FaMYB1-OE* strawberry fruits. *A* - relative expression of *FaMYB1* in *FaMYB1-RNAi* (*black bar*), *FaMYB1-OE* (*grey bar*), and fruits infiltrated with a control vector (*white bar*) were detected using the qRT-PCR and normalized against *FaGAPDH*. The normalized expression for controls was set to 1. Means ± SD, n = 4 - 5. *B* - Phenotype of *FaMYB1-RNAi* fruits, *C* - phenotype of the *FaMYB1-OE* fruits, *D* - phenotype of the controls. *E* - Anthocyanin content was measured in *FaMYB1-RNAi* (*black bar*), *FaMYB1-OE* (*grey bar*), and in controls (*white bars*) n = 4 - 5. *Asterisks* indicate significant differences between the *FaMYB1-RNAi* or *FaMYB1-OE* fruits and the controls (one-way *ANOVA* with post-hoc Dunnet's test; * - $P < 0.05$, *** - $P < 0.001$).

The FaMYB1 function in strawberry fruits was examined by overexpressing or down-regulating *FaMYB1 in planta* using *Agrobacterium*-mediated transformations. The *FaMYB1* overexpression induced a significant decrease in anthocyanin content, and also a significant decrease in *FaANS* and *FaFGT* expression. In a tobacco model, Aharoni *et al.* (2001) reported that *FaMYB1* overexpression is associated with a decreased *ANS* expression and FGT activity leading to a reduced amount of anthocyanins in flowers, which is consistent with our results. Furthermore, an impairment of *ANS* and *FGT* genes causes anthocyanin content reductions in strawberry and other plant species (Kim *et al.* 2005, Griesser *et al.* 2008, Debes *et al.* 2011, Zhao *et al.* 2012). In contrast, the *FaDFR1* expression increased compared to the control. The *FaDFR1* up-regulation might give rise to increased *FaANS* and *FaLAR* substrates. However, anthocyanin synthesis did not accelerate due to a decreased *FaANS* expression. Therefore, reductions in *FaANS* and *FaFGT* expression might be the primary cause for a decreased anthocyanin content in the *FaMYB1-OE* fruits.

The siRNA-mediated *FaMYB1* down-regulation resulted in a significant increase in anthocyanin content compared to the control fruits. However, the expressions of assayed flavonoid pathway genes were not affected with the exception of *FaF3H*. In the *FaMYB1-RNAi* fruits, the anthocyanin content increased, therefore the *FaF3H* step might not be limiting for anthocyanin biosynthesis. In the *FaMYB1-RNAi* fruits, *FaLAR* was also reduced. The *FaLAR* is directly involved in PA biosynthesis and uses leucocyanidin and leucopelargonidin as substrates, as well as *FaANS*. The recombinant enzyme analysis of flavonoid pathway genes shows 4'-hydroxylated flavonoids were preferred substrates for FaF3H, FaDFR1, and FaANS, whereas FaLAR and FaANR exhibited higher activities with 3',4'-hydroxylated compounds. Notably, a strict substrate specificity was detected for only FaLAR which used leucocyanidin, a 3',4'-hydroxylated compound (Almeida *et al.* 2007). The 3'-hydroxylation of phenolic compounds is catalyzed by flavonoid-3'-hydroxylase (F3'H). An *FaF3'H* expression is highest during early stages, and markedly decreases at the stages following Wh (Carbone *et al.* 2009). More recently, Salvatierra *et al.* (2013) has identified *FcMYB1* which encodes a *FaMYB1* ortholog from *F. chiloensis*. When fully ripe, a pinkish-white receptacle and red achenes characterize this wild strawberry species. An *FcMYB1* down-regulation in *F. chiloensis* white fruits leads to an increased pigmentation relative to control fruits, which is accompanied by an increased *ANS* and *FGT* transcription and a substantial decrease in *LAR* and *ANR* transcription. Together, the reduction in *FaLAR* expression and the increase in non-preferred substrates for FaLAR (leucopelargonidin) and FaANR (pelargonidin) might cause the decrease in PA biosynthesis resulting in redirection of flavonoid precursor metabolism. This process could increase anthocyanin accumulation at the expense of PA biosynthesis, which might explain the anthocyanin accumulation observed in the *FaMYB1-RNAi* fruits. Similarly, Fischer *et al.* (2013) showed that the PA pathway in strawberry fruits is affected by down-regulation of the *ANR* gene leading to ectopic anthocyanin biosynthesis.

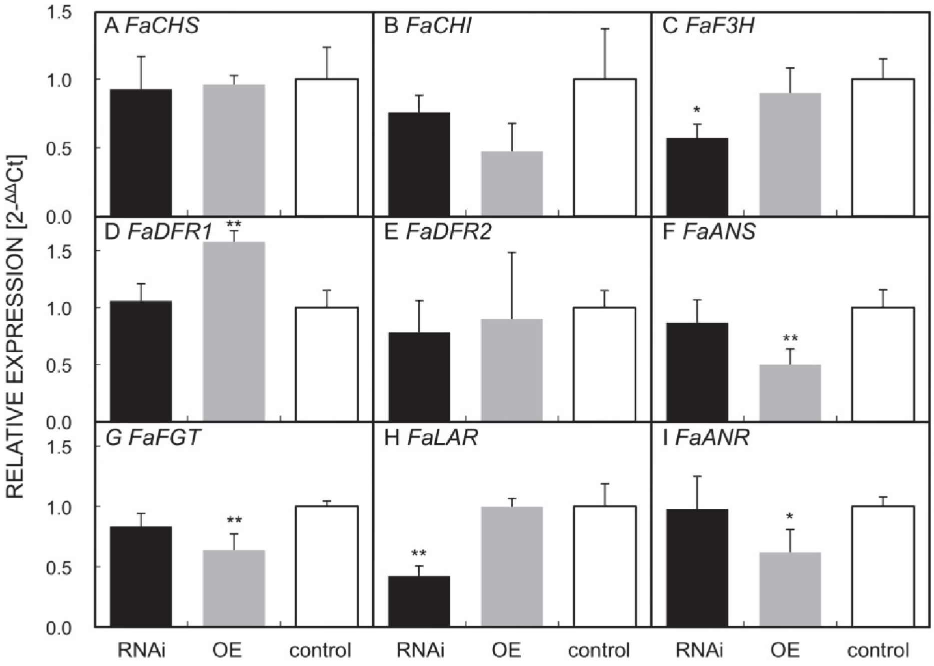

Fig. 6. Flavonoid pathway genes profiling for *FaMYB1-RNAi* and *FaMYB1-OE* strawberry fruits. Expression of *FaCHS* (*A*), *FaCHI* (*B*), *FaF3H* (*C*), *FaDFR1* (*D*), *FaDFR2* (*E*), *FaANS* (*F*), *FaFGT*, (*G*) *FaLAR* (*H*), and *FaANR* (*I*) was detected using qRT-PCR. Relative expression for each gene was normalized to the *FaGAPDH* expression, normalized expression of each gene in the control was set to 1. Means ± SD, *n* = 3 - 5. *Asterisks* indicate significant differences between *FaMYB1-RNAi* (*black bars*) or *FaMYB1-OE* (*grey bars*) fruits and control fruits (*white bars*) (one-way ANOVA with post-hoc Dunnet's test; * - $P < 0.05$, ** - $P < 0.01$).

In contrast to our results, Medina-Puche *et al.* (2014) has reported that the anthocyanin content of *FaMYB1*-silenced fruit receptacles (cv. Elsanta) remains unchanged although *FaMYB1* transcription is notable reduced. The following three minimum differences were observed between Medina-Puche *et al.* (2014) and the present study: *1*) the transformation methods; *2*) the cultivars used; and *3*) the *Agrobacterium* strains employed. Further, Medina-Puche *et al.* used fruits attached to a plant, and we used post-harvest fruits. Therefore, a fruit condition may influence interaction between FaMYB1 and cofactors, such as WD40 and bHLH proteins, resulting in effects on downstream gene expression. Collectively, the methodology could affect the differences in observed results between the studies.

Our results demonstrate that *FaMYB1* was highly expressed in fully mature red fruits, but *FaMYB1* overexpression produced only pink-colored fruits. Thus, the discrepancy between the *FaMYB1* expression patterns and FaMYB1 function in fruit coloration were observed. Transcription of *FaMYB10,* a well-known strong positive regulator of anthocyanin biosynthesis, is low until fruits reach the full size. A nearly 40 000-fold increase in its relative transcription occurs at fruit ripening and a colour change (Lin-Wang *et al.* 2010). We also determined *FaMYB10* expression patterns during strawberry fruit ripening, and *FaMYB10* showed an expression similar to the published results (Fig. 1 Suppl.). Although *FaMYB1* transcription showed two phases, the variation in *FaMYB1* transcription was much lower than in *FaMYB10*. Based on these reports and our results, we conclude that FaMYB1 might be dominant and repressed anthocyanin biosynthesis at the SG stage. Although the *FaMYB1* expression was higher at the Re stage than in the other stages, FaMYB10 was dominant due to its strong induction. Therefore, anthocyanin biosynthesis was accelerated at Re. However, detailed FaMYB1 and FaMYB10 regulation mechanisms require further studies.

In conclusion, the results of this study demonstrate that *FaMYB1* expression was regulated by radiation, and *FaMYB1* might negatively regulate anthocyanin biosynthesis at the branching-point of the anthocyanin/PA biosynthesis in strawberry fruits.

References

Aharoni, A., De Vos, C.H., Wein, M., Sun, Z., Greco, R., Kroon, A., Mol, J.N., O'Connell, A.P.: The strawberry *FaMYB1* transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. - Plant J. **28**: 319-332, 2001.

Alexander, L., Grierson, D.: Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. - J. exp. Bot. **53**: 2039-2055, 2002.

Almeida, J.R., D'Amico, E., Preuss, A., Carbone, F., De Vos, C.H., Deiml, B., Mourgues, F., Perrotta, G., Fischer, T.C., Bovy, A.G., Martens, S., Rosati, C.: Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry (*Fragaria* × *ananassa*). - Arch. Biochem. Biophys. **465**: 61-71, 2007.

Anttonen, M.J., Hoppula, K.I., Nestby, R., Verheul, M.J., Karjalainen, R.O.: Influence of fertilization, mulch, color, early forcing, fruit order, planting date, shading, growing environment, and genotype on the contents of selected phenolics in strawberry (*Fragaria* × *ananassa* Duch.) fruits. - J. agr. Food Chem. **54**: 2614-2620, 2006.

Azuma, A., Yakushiji, H., Koshita, Y., Kobayashi, S.: Flavonoid biosynthesis-related genes in grape skin are differentially regulated by temperature and light conditions. - Planta **236**: 1067-1080, 2012.

Baudry, A., Heim, M.A., Dubreucq, B., Caboche, M., Weisshaar, B., Lepiniec, L.: TT2, TT8, and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. - Plant J. **39**: 366-380, 2004.

Carbone, F., Preuss, A., De Vos, R.C., D'Amico, E., Perrotta, G., Bovy, A.G., Martens, S., Rosati, C.: Developmental, genetic and environmental factors affect the expression of flavonoid genes, enzymes and metabolites in strawberry fruits. - Plant Cell Environ. **32**: 1117-1131, 2009.

Chai, Y.M., Jia, H.F., Li, C.L., Dong, Q.H., Shen, Y.Y.: FaPYR1 is involved in strawberry fruit ripening. - J. exp. Bot. **62**: 5079-5089, 2011.

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

Debes, M.A., Arias, M.E., Grellet-Bournonville, C.F., Wulff, A.F., Martínez-Zamora, M.G., Castagnaro, A.P., Díaz-Ricci, J.C.: White-fruited *Duchesnea indica* (Rosaceae) is impaired in *ANS* gene expression. - Amer. J. Bot. **98**: 2077-2083, 2011.

Dussi, M.C., Sugar, D., Wrolstad, R.E.: Characterizing and quantifying anthocyanins in red pears and the effect of light quality on fruit color. - Amer. Soc. hort. Sci. **120**: 785-789, 1995.

Fait, A., Hanhineva, K., Beleggia, R., Dai, N., Rogachev, I., Nikiforova, V.J., Fernie, A.R., Aharoni, A.: Reconfiguration of the achene and receptacle metabolic networks during strawberry fruit development. - Plant Physiol. **148**: 730-750, 2008.

Fischer, T.C., Mirbeth, B., Rentsch, J., Sutter, C., Ring, L., Flachowsky, H., Habegger, R., Hoffmann, T., Hanke, M.V., Schwab, W.: Premature and ectopic anthocyanin formation by silencing of anthocyanidin reductase in strawberry (*Fragaria* × *ananassa*). - New Phytol. **201**: 440-451, 2014.

Griesser, M., Hoffmann, T., Bellido, M.L., Rosati, C., Fink, B., Kurtzer, R., Aharoni, A., Muñoz-Blanco, J., Schwab, W.: Redirection of flavonoid biosynthesis through the down-regulation of an anthocyanidin glucosyltransferase in ripening strawberry fruit. - Plant Physiol. **146**: 1528-1539, 2008.

Hoffmann, T., Kalinowski, G., Schwab, W.: RNAi-induced silencing of gene expression in strawberry fruit (*Fragaria* × *ananassa*) by agroinfiltration: a rapid assay for gene function analysis. - Plant J. **48**: 818-826, 2006.

Jia, H.J., Araki, A., Okamoto, G.: Influence of fruit bagging on aroma volatiles and skin coloration of "Hakuho" peach (*Prunus persica* Batsch). - Postharvest Biol. Technol. **35**: 61-68, 2005.

Jia, H.F., Chai, Y.M., Li, C.L., Lu, D., Luo, J.J., Qin, L., Shen, Y.Y.: Abscisic acid plays an important role in the regulation of strawberry fruit ripening. - Plant Physiol. **157**: 188-199, 2011.

Jia, H.F., Lu, D., Sun, J.H., L,i C.L., Xing, Y., Qin, L., Shen, Y.Y.: Type 2C protein phosphatase ABI1 is a negative regulator of strawberry fruit ripening. - J. exp. Bot. **64**: 1677-1687, 2013.

Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtens, F., Jones, J., Tonelli, C., Weisshaar, B., Martin, C.: Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. - EMBO J. **19**: 6150-

6161, 2000.

Josuttis, M., Dietrich, H., Treutter, D., Will, F., Linnemannstöns, L., Krüger, E.: Solar UV-B response of bioactives in strawberry (*Fragaria* × *ananassa* Duch. L.): a comparison of protected and open-field cultivation. - J. agr. Food Chem. **58**: 12692-12702, 2010.

Kadomura-Ishikawa, Y., Miyawaki, K., Noji, S., Takahashi, A.: *Phototropin 2* is involved in blue light-induced anthocyanin accumulation in *Fragaria* × *ananassa* fruits. - J. Plant Res. **126**: 847-857, 2013.

Kami, C., Lorrain, S., Hornitschek, P., Fankhauser, C.: Light-regulated plant growth and development. - Curr. Topics dev. Biol. **91**: 29-66, 2010.

Kataoka, I., Beppu, K.: UV irradiance increases development of red skin color and anthocyanins in “Hakuho” peach. - HortScience **39**: 1234–1237, 2004.

Kim, S., Jones, R., Yoo, K.S., Pike, L.M.: The *L* locus, one of complementary genes required for anthocyanin production in onions (*Allium cepa*), encodes anthocyanidin synthase. - Theor. appl. Genet. **111**: 120-127, 2005.

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

Klee, H.J., Giovannoni, J.J.: Genetics and control of tomato fruit ripening and quality attributes. - Annu. Rev. Genet. **45**: 41-59, 2011.

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

Kortstee, A.J., Khan, S.A., Helderman, C., Trindade, L.M., Wu, Y., Visser, R.G., Brendolise, C., Allan, A., Schouten, H.J., Jacobsen E.: Anthocyanin production as a potential visual selection marker during plant transformation. - Transgenic Res. **20**: 1253-1264, 2011.

Lin, W.K., Bolitho, K., Grafton, K., Kortstee, A., Karunairetnam, S., McGhie, T.K., Espley, R.V., Hellens, R.P, Allan, A.C.: An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in *Rosaceae*. - BMC Plant Biol. **21**:10:50, 2010.

Miyawaki, K., Fukuoka, S., Kadomura, Y., Hamaoka, H., Mito, T., Ohuchi, H., Schwab, W., Noji, S.: Establishment of a novel system to elucidate the mechanisms underlying light-induced ripening of strawberry fruit with an *Agrobacterium*-mediated RNAi technique. - Plant Biotechnol. **29**: 271-277, 2012.

Medina-Puche, L., Cumplido-Laso, G., Amil-Ruiz, F., Hoffmann, T., Ring, L., Rodríguez-Franco, A., Caballero, J.L., Schwab, W., Muñoz-Blanco, J., Blanco-Portales, R.: *MYB10* plays a major role in the regulation of flavonoid/phenylpropanoid metabolism during ripening of *Fragaria* × *ananassa* fruits. - J. exp. Bot. **65**: 401-417, 2014.

Osorio, S., Scossa, F., Fernie, A.R.: Molecular regulation of fruit ripening. - Front. Plant Sci. **4**: 198, 2013.

Paolocci, F., Robbins, M.P., Passeri, V., Hauck, B., Morris, P., Rubini, A., Arcioni, S., Damiani, F.: The strawberry transcription factor *FaMYB1* inhibits the biosynthesis of proanthocyanidins in *Lotus corniculatus* leaves. - J. exp. Bot. **62**: 1189-1200, 2011.

Salvatierra, A., Pimentel, P., Moya-Leon, M.A., Caligari, P.D., Herrera, R.: Comparison of transcriptional profiles of flavonoid genes and anthocyanin contents during fruit development of two botanical forms of *Fragaria chiloensis* ssp. *chiloensis*. - Phytochemistry **71**: 1839-1847, 2010.

Salvatierra, A., Pimentel, P., Moya-León, M.A., Herrera, R.: Increased accumulation of anthocyanins in *Fragaria chiloensis* fruits by transient suppression of *FcMYB1* gene. - Phytochemistry **90**: 25-36, 2013.

Tamagnone L., Merida A., Parr A., Mackay S., Culianez-Macia F.A., Roberts K., Martin C.: The AmMYB308 and AmMYB330 transcription factors from *Antirrhinum* regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. - Plant Cell **10**: 135-154, 1998.

Uleberg, E., Rohloff, J., Jaakola, L., Trôst, K., Junttila, O., Häggman, H., Martinussen, I.: Effects of temperature and photoperiod on yield and chemical composition of northern and southern clones of bilberry (*Vaccinium myrtillus* L.). - J. agr. Food Chem. **60**: 10406-10414, 2012.

Vogt, T.: Phenylpropanoid biosynthesis. - Mol. Plant **3**: 2-20, 2010.

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

Wei, Y.Z., Hu, F.C., Hu, G.B., Li, X.J., Huang, X.M., Wang, H.C.: Differential expression of anthocyanin biosynthetic genes in relation to anthocyanin accumulation in the pericarp of *Litchi chinensis* Sonn. - PLoS One **6**: e19455, 2011.

Zhao, Z.C., Hu, G.B., Hu, F.C., Wang, H.C., Yang, Z.Y., Lai, B.: The UDP glucose: flavonoid-3-*O*-glucosyltransferase (*UFGT*) gene regulates anthocyanin biosynthesis in litchi (*Litchi chinesis* Sonn.) during fruit coloration. - Mol. Biol. Rep. **39**: 6409-6415, 2012.

Zhou Y., Singh B.R.: Effect of light on anthocyanin levels in submerged, harvested cranberry fruit. - J. Biomed. Biotechnol. **2004**: 259-263, 2004.