Journal of Experimental Botany, Vol. 65, No. 2, pp. 401–417, 2014
doi:10.1093/jxb/ert377 Advance Access publication 25 November, 2013

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

MYB10 plays a major role in the regulation of flavonoid/phenylpropanoid metabolism during ripening of *Fragaria* × *ananassa* fruits

Laura Medina-Puche[1,*], Guadalupe Cumplido-Laso[1,*], Francisco Amil-Ruiz[1], Thomas Hoffmann[2], Ludwig Ring[2], Antonio Rodríguez-Franco[1], José Luis Caballero[1], Wilfried Schwab[2], Juan Muñoz-Blanco[1,†] and Rosario Blanco-Portales[1]

[1] Departamento de Bioquímica y Biología Molecular. Edificio Severo Ochoa, Campus Universitario de Rabanales y Campus de Excelencia Internacional Agroalimentario CEIA3, Universidad de Córdoba, 14071 Córdoba, Spain
[2] Biotechnology of Natural Products, Technische Universität München, Liesel-Beckmann-Str. 1, 85354 Freising, Germany

* These authors contributed equally to this manuscript.
† To whom correspondence should be addressed. E-mail: bb1mublj@uco.es

Received 9 August 2013; Revised 9 October 2013; Accepted 14 October 2013

Abstract

This work characterized the role of the R2R3-MYB10 transcription factor (TF) in strawberry fruit ripening. The expression of this TF takes place mainly in the fruit receptacle and is repressed by auxins and activated by abscisic acid (ABA), in parallel to the ripening process. Anthocyanin was not produced when *FaMYB10* expression was transiently silenced in fruit receptacles. An increase in *FaMYB10* expression was observed in water-stressed fruits, which was accompanied by an increase in both ABA and anthocyanin content. High-throughput transcriptomic analyses performed in fruits with downregulated *FaMYB10* expression indicated that this TF regulates the expression of most of the Early-regulated Biosynthesis Genes (*EBGs*) and the Late-regulated Biosynthesis Genes (*LBGs*) genes involved in anthocyanin production in ripened fruit receptacles. Besides, the expression of *FaMYB10* was not regulated by FaMYB1 and vice versa. Taken together, all these data clearly indicate that the *Fragaria* × *ananassa* MYB10 TF plays a general regulatory role in the flavonoid/phenylpropanoid pathway during the ripening of strawberry

Key words: Anthocyanins, flavonoid/phenylpropanoid biosynthesis, fruit receptacle, ripening, strawberry, transcription factor.

Introduction

The accumulation of anthocyanin pigments in fruits is an important indicator of ripeness and fruit quality. Pigments influence the attraction of pollinators and predators contributing to seed dispersal, protect against UV light damage and pathogen attack, and play an important role in plant protection against biotic and abiotic stresses (Feild *et al.*, 2001; Regan *et al.*, 2001; Winkel-Shirley, 2001; Schaefer *et al.*, 2004; Tanaka *et al.*, 2008). In strawberries, the red colouration of fruit receptacles is mainly due to anthocyanin accumulation, which plays a major role in consumer preference and marketability (Perkins-Veazie, 1995; Chiu *et al.*, 2010; Carvalho *et al.*, 2010; Lin-Wang *et al.*, 2010; Yang *et al.*, 2010; Hichri *et al.*, 2011).

The flavonoid/phenylpropanoid (F/P) pathway in plants is responsible for anthocyanin biosynthesis. These pathways are organized as metabolic branches that account for the synthesis of many different compounds, such as hydroxycinnamic acid, isoflavones, flavonols, phlobaphenes, and pro-anthocyanidins. They are responsible for the major red, purple, violet, and blue pigments found in many flowers and fruits (Petroni and Tonelli, 2011).

Many highly conserved transcription factors (TFs) regulate the expression of the genes involved in F/P metabolism. They are mainly R2R3-MYB TFs interacting or not with basic helix–loop–helix proteins and/or with proteins containing

conserved WD40 repeats to form the so-called ternary MBW complexes (Hichri *et al.*, 2011; Petroni and Tonelli, 2011). To date, there are documented cases of individual genes or even branches of F/P metabolism that are regulated by these TFs (Hichri *et al.*, 2011; Petroni and Tonelli, 2011). Thus, in maize, the genes responsible for anthocyanin biosynthesis are activated as a single unit by a unique MBW complex. In *Arabidopsis*, there is an independent co-activator, R2R3-MYB TF, lacking both basic helix–loop–helix and WD40 that regulates the expression of the Early-regulated Biosynthesis group of Genes (*EBGs*), as well as a MBW complex regulating the expression of the Late-regulated Biosynthesis group of Genes (*LBGs*; Petroni and Tonelli, 2011). In certain species such as apple and grape, anthocyanin biosynthesis is regulated mainly by MYB–basic helix–loop–helix complexes lacking WD40 (Takos *et al.*, 2006; Ban *et al.*, 2007; Espley *et al.*, 2007). In grapes (*Vitis vinifera*), the anthocyanin biosynthesis branch is controlled by a single locus containing four MYB genes. Two of them, known as VvMYBA1 and VvMYBA2 respectively, are involved in skin colour regulation (Kobayashi *et al.*, 2004; Cutanda-Perez *et al.*, 2009). Mutations in the promoter region of VvMYBA1 or in the coding region of VvMYBA2 lead to a loss of anthocyanin biosynthesis in the skin, thus rendering grapes white (Kobayashi *et al.*, 2004). In apple (*Malus* × *domestica*), there are three different alleles of *MYB* (*MYB10*, *MYB1*, and *MYBA*) controlling the red pigmentation of the fruit (Takos *et al.*, 2006; Ban *et al.*, 2007; Chagne *et al.*, 2007; Lin-Wang *et al.*, 2010). While MYB1 and MYBA are involved in anthocyanin biosynthesis in the skin, MYB10 is involved in both the skin and the flesh (Takos *et al.*, 2006; Ban *et al.*, 2007). In nectarine, MYB10 positively regulates UDP glucose:flavonoid-3-*O*-glucosyltransferase (*UFGT*) and dihydroflavonol 4-reductase (*DFR*) promoters (Ravaglia *et al.*, 2013). Recently, it has also been shown that the expression profile of *MYB10* putative orthologues found in 20 different *Rosaceous* fruits, including strawberry, correlated with anthocyanin production throughout the fruit-ripening process, and that this required the presence of specific basic helix–loop–helix proteins (Espley *et al.*, 2007; Lin-Wang *et al.*, 2010). In tomato and pepper, certain R2R3-MYB genes, such as LeMYB12, LeANT1, LeAN2, and CaA, also regulate anthocyanin and flavonol biosynthesis (reviewed by Petroni and Tonelli, 2011). In general, it has been reported that these TFs activate the expression of LBGs during fruit development (Borovsky *et al.*, 2004). Several R2R3-MYB TFs, such as FaMYB1, also seem to act as phenylpropanoid and anthocyanin pathway repressors (Aharoni *et al.*, 2001).

Most of the abovementioned studies represent cases showing that one of several of these MYB TFs regulates either a particular branch of genes involved in F/P metabolism or is required for the expression of any or some of these genes in a determined tissue. The cases describing TFs playing a general or a broader regulatory role in F/P metabolism are scarce, and so are those analysing the overall transcriptomic changes that take place during this process. Exceptions are the maize and the *VvMYB5a*/*5b* genes that play a general regulatory role in *V. vinifera*, since apparently they can simultaneously control the expression of all branches of F/P metabolism, including those involved in anthocyanin biosynthesis (Deluc *et al.*, 2006). Strawberry transgenic plants harbouring 35S:*FaMYB10* were obtained by Lin-Wang *et al.* (2010). In these plants, the increase of *FaMYB10* expression caused the synthesis of high levels of anthocyanins. Recently, similar results were observed in apple transgenic plants overexpressing *MYB10*. Thus, *MYB10* overexpression in these plants led to an increase of anthocyanins and concentrations of flavonoids and proanthocyanins (Espley *et al.*, 2013)

This work presents the functional and molecular characterization of the *Fragaria* gene *FaMYB10*. High-throughput transcriptomic analyses performed in ripened fruits with the expression of downregulated *FaMYB10* clearly show that this TF is a direct general regulator of *EBG* and *LBG* corresponding to the F/P pathway, including those involved in anthocyanin production. These transcriptomic studies virtually preclude the involvement of many other different MYB genes in this regulation. This work also shows that the expression of *FaMYB10* takes place mainly in the receptacle. Besides, the expression of this gene as well as that of those genes regulated by this TF are also clearly under the control of hormones being repressed by auxins and induced by abscisic acid (ABA), which accounts for the molecular bases of the recently described role of this latter hormone in the strawberry ripening process (Chai *et al.*, 2011; Jia *et al.*, 2011).

Materials and methods

Plant material

Strawberry plants (*Fragaria* × *ananassa* Duch. cv. Camarosa, an octoploid cultivar) were grown under field conditions in Huelva, southwest Spain). *Fragaria* × *ananassa* fruits were harvested at different developmental stages: small-sized green fruits (G1, 2–3 g), full-sized green fruits (G3, 4–7 g), white fruits (W, 5–8 g), full-ripe red fruits (R, 6–10 g), overripe fruits (OR, 6–10 g), and senescent fruits (SN, 6–10 g). Vegetative tissues, such as runners, roots, crowns, and expanding leaves were also harvested. All analysed tissues were immediately frozen in liquid nitrogen after harvesting and stored at –80 °C. Strawberry plants (*Fragaria* × *ananassa* Duch. cv. Elsanta) used for agroinfiltration were grown in plant chamber at 25 °C, 10 000 lux, and 80% humidity and afterwards maintained in a greenhouse.

Auxin treatment

Achenes of two sets of 50 middle-sized green fruits (G2) each, still attached to the plant, were carefully removed using the tip of a scalpel blade. One set of de-achened G2 fruits were treated with the synthetic auxin 1-naphthaleneacetic acid in lanolin paste (1 ml) with 1 mM 1-naphthaleneacetic acid in 1% (w/v) dimethyl sulphoxide. The other set of G2 de-achened fruits (control group) were treated with the same paste, but without 1-naphthaleneacetic acid. Both treatments were applied over the entire fruit surface. All fruits were harvested 5 d after treatment, immediately frozen in liquid nitrogen, and stored at –80 °C. For assay, frozen fruits were ground with mortar and pestle in the presence of liquid nitrogen. Over the course of the assays, the fruits reached the G3–W developmental stage.

Nordihydroguaiaretic acid treatment

Nordihydroguaiaretic acid (NDGA) is an ideal inhibitor of the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED) and was used to block ABA biosynthesis (Creelman *et al.*, 1992). The lowest

NDGA concentration which completely blocks ABA accumulation is 100 µM, as determined by preliminary tests in tomato fruit (Zhang *et al.*, 2009). Strawberry fruits (*Fragaria* × *ananassa* cv. Elsanta) were used at the mature G–W stages for the purposes of this study. All fruits (20 fruits in total) were carefully injected with 1–2 ml of NDGA (100µM) sterile solution or sterile water (control fruits) using a hypodermic syringe. Three replications were conducted for each treatment. The samples were harvested after 8 d of treatment, when the fruits reached the R developmental stage, frozen in liquid nitrogen, and stored at –80 °C. Fruits treated with NDGA were white, while control fruits showed a red phenotype. These samples were used for ABA content measurement and relative expression of *FaMYB10*.

Water stress treatment

Fruits at the G–W stages from Fragaria × ananassa cv. Elsanta were used in this experiment. The control fruits maintained pedicels immersed in MS medium with sucrose (renewed every 2 d), while the fruits under water stress retained air pedicels without MS medium. The fruits were maintained at 25 °C under a 16/8 light/dark cycle with 120 µmol m^{-2} s^{-1} irradiance (Osram Fluora lamps, München, Germany) and the pedicels of all fruits were cut daily. The samples were harvested 4 d after treatment, frozen in liquid nitrogen, and stored at –80 °C until RNA extraction. The water-stressed fruits were red, while the control fruits showed a white phenotype.

Preparation of deuterated ABA

Deuterated ABA was used as an internal standard. In order to prepare the standard, the ring protons of ABA (5 mg) were exchanged over 48 h at room temperature in 10 ml heavy water (isotopic purity 99.96%, Sigma) in presence of 1.0 M deuterated sodium hydroxide (minimum isotopic purity 99%, Sigma), in accordance with Rock and Zeevaart (1990). The medium was acidified with hydrochloric acid to pH 3, and the acidic solution was partitioned three times against equal volumes of diethyl ether. The ether phases were subsequently combined and dried under vacuum at 35 °C, and the samples containing [H] were dissolved in methanol.

ABA extraction procedure

The purity of [$^2H_6$]ABA obtained by HPLC was assessed by mixing 1 g powdered material with 1.26 nmol of internal standard (40 µl of 31.5 nmol ml^{-1} intermediate standard solution) for 5 min. The sample was extracted twice with 10 ml methanol/water (1:1, v/v, pH 5.5) and the mixture was centrifuged at 5000 *g* for 5 min at room temperature. The supernatant was extracted twice with dichloromethane (10 ml) and the extracts obtained were centrifuged again at 5000 *g* at room temperature for 5 min; the lower phase was evaporated under vacuum conditions at 40 °C. The residue was dissolved in 100 µl of 100% acetone and 250 µl of 0.1% formic acid in water/acetonitrile (70:30, v/v). Lastly, the sample was centrifuged at full speed for 5 min and the supernatant obtained was used for analysis.

Total anthocyanidin analysis

Approximately 0.3 g of the resultant powder was poured into 3 ml of 1% (v/v) hydrochloric acid/methanol and kept at 0 °C for 10 min. The slurry was centrifuged at 1500 *g* and 4 °C for 10 min. The supernatant was saved and its absorbance at 515 nm was measured. The amount of anthocyanins was calculated by using E_{molar} = 36 000 l mol^{-1} cm^{-1} (Woodward, 1972; Bustamante *et al.*, 2009). Three replicates were performed per each analysed treatment.

HPLC-MS quantitative analysis of ABA

The amount of ABA in strawberry fruits treated with NDGA was determined using an HPLC-MS system (VARIAN 1200L Triple Quadrupole) with a column (150 × 2.1 mm i.d. Phenomenex C_{18} with 3 µm particle, California, USA). The injection volume was 8 µl, and the mobile phases used were water/0.1% formic acid (A) and acetonitrile/methanol (75:25, v/v; (B) with gradient program: start with 95:5 A/B, 2 min with 65/35 A/B, 10 min with 0/100 A/B, and holding at 15 min with 0/100 A/B, at a constant flow rate of 0.2 ml min^{-1}. The MS was operated in the negative selecting ion monitoring mode. The source was operated with N_2 (LCMS quality) at 344.74 kPa. MS parameters were optimized by flow injection analysis of the individual solutions of ABA and [$^2H_6$]ABA. These compounds gave a response in the selecting ion monitoring interface in the negative mode but not in the positive mode. The used ESI needle voltage was –5.5 kV. Other optimized conditions for MS included: drying gas 270 °C; source temperature 55 °C; capillary voltage 45 V; shield voltage 600 V; detector voltage 1500 V, and dwell time 1 s per scan. Quadruple 1-ion resolution was optimized at 0.5 Da. The quantitative data obtained from calibration standards and plant samples were processed using Work Station software (Varian, California, USA).

Metabolite extraction

Fruits were individually frozen, lyophilized (Christ RVC 2–18) for 24 h, and homogenized with a mill (Retsch MM 200, Haan, Germany) to a fine powder. An aliquot of 50 mg lyophilized fruit powder was used for each of the five biological replicates. Biochanin A (250 µl of a solution in methanol, 0.2 mg ml^{-1}) was added as an internal standard, yielding 50 µg internal standard in each sample. After addition of 250 µl, methanol, vortexing and sonication (Bandelin Sonopuls GM 2017) for 10 min, the sample was centrifuged at 16 000 *g* for 10 min. The supernatant was removed and the residue re-extracted with 500 µl methanol. The supernatants were combined, concentrated to dryness in a vacuum concentrator and redissolved in 35 µl water. After 1-min vortexing, 10-min sonication, and 10-min centrifugation at 16 000 g, the clear supernatant was used for LC/MS-analysis. Each extract was injected twice (technical replicate).

LC-ESI-MS^n analysis of phenolic metabolites

Metabolites samples were analysed on a 1100 HPLC/UV-system (Agilent Technologies, Waldbronn, Germany) equipped with a reversed-phase column (Luna 3u C18(2) 100A 150 × 2mm, Phenomenex, Aschaffenburg, Germany) and connected to a Bruker esquire3000^{plus} ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Mobile phases 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) were used. Injection volume was 5 µl and flow rate was 0.2 ml min^{-1}. A gradient was applied which started at 0% B and rose to 50% B in 30 min. Within the next 5 min, B was increased to 100%, where it remained for 15 min. Afterwards, B was decreased to 0% within 5 min. These initial conditions were kept for 10 min for system equilibration. UV signals were detected at 280 nm. MS spectra were recorded in alternating polarity mode. Nitrogen was used as nebulizer gas at 206.84 kPa and as dry gas at 330 °C and 9 l min^{-1}. The electrospray ionization voltage of the capillary was set to –4000 V, the end plate to –500 V, the skimmer to 40 V, and the capillary exit to 121 V. The full scan ranged from 100 to 800 m/z with a resolution of 13 000 m/z s^{-1}. Ions were accumulated until an ion charge count) target of 20 000 (positive mode) and 10 000 (negative mode), respectively, was achieved or the maximum time of 200 ms was reached. For tandem mass spectrometry, helium was used as collision gas at 4×10^{-6} mbar and a collision voltage of 1 V. Data was analysed with Data Analysis 5.1 software (Bruker Daltonics, Bremen, Germany). Metabolites were identified by comparing their retention time and mass spectra (MS and MS^2) with that of measured reference compounds. Statistical evaluation was performed using independent Student's t-test of SOFA Statistics (Paton-Simpson and Associates, Auckland, NZ).

RNA isolation

Total RNA was isolated from independent pools of strawberry fruits at different growth and ripening stages and vegetative tissues, as stated by Asif *et al.* (2000). Achenes were always removed from fruit samples and only receptacle RNA was extracted and purified. The total RNA obtained was treated with DNase I (RNase free, Invitrogen), in accordance with the manufacturer's instructions, to remove genomic DNA contamination, after which it was purified by using a RNeasy Mini kit (Qiagen). RNA samples were considered DNA free when no amplicons corresponding to the analysed genes were observed, using RNA as a template for a standard PCR reaction.

Microarray generation and analysis

This work compared the cDNA sequences contained in Kevin Folta's libraries (Shulaev *et al.*, 2011) against those in this group's own cDNA libraries (Bombarely *et al.*, 2010). The percentage of identity between the cDNA sequences from *Fragaria* × *ananassa* and those from *Fragaria vesca* was always over 98.6% and similar to that previously found (Bombarely *et al.*, 2010). This work subsequently decided to generate a custom-made oligo-based (60-mer length) microarray platform containing a total of 35 234 singletons corresponding to those sequences published in the strawberry genome project (http://www.strawberry.org).

For each sequence, four different oligos were printed per block and four blocks were printed per data set. Samples of total RNA were DNaseI-treated and afterwards purified by Qiagen columns, in accordance with manufacturer conditions. Sample labelling (Cy3), hybridization with four probes for target, and data normalization were performed by Nimblegen Systems, in accordance with the procedures described in its expression analysis section (http://www.nimblegen.com/). Briefly, 10 µg total receptacle RNA were processed using the Roche cDNA Synthesis System optimized for double-stranded cDNA synthesis. The cDNA was purified using High Pure PCR Product Purification, in accordance with the kit's protocol. Samples were then processed using full-size reverse-transcription reactions. Three replicate reverse-transcription reactions were performed for each total RNA input amount. Each cDNA sample (1 µg) was random-primer labelled with Cy3-nonamers using NimbleGen One-Color DNA Labeling Kits, in accordance with Roche NimbleGen's standard protocol. Using random assignment, each Cy3-labelled cDNA sample was applied to custom-made strawberry 12 × 135K array formats (each slide contains 12 independent arrays, each with 140 856 probes covering 35 214 genes, four probes/target gene). The array was then hybridized for 16 h at 42°C, washed, dried, and scanned at 2-µm resolution using a NimbleGen MS 200 Microarray Scanner (Roche NimbleGen). NimbleScan version 2.6 software was used to extract fluorescence intensity signals from the scanned images and perform robust multi-array analyses to generate gene expression values. The analyses were performed across replicate arrays within each test condition, sample and input amount (for example, separate analyses were performed for the datasets from the three replicate hybridizations using cDNA derived from 10 µg total RNA).

Data analysis of the microarray expression studies was performed with the software for gene expression analysis Array Star (DNASTAR). Moderated t-test and false discovery rate (Benjamini and Hochberg, 1995) for multiple testing corrections were used with $P < 0.01$ to statistically identify significant differences.

Validation of microarray data and expression analysis by quantitative real-time PCR

Expression analyses of *FaMYB10* (EU155162) and the genes herein analysed and studied in all physiological conditions were performed by quantitative real-time PCR (qRT-PCR) using a iCycler system (BioRad), as previously described (Benítez-Burraco *et al.*, 2003). First-strand cDNA was obtained using 2 µg total RNA and a iScript kit (BioRad), in accordance with the manufacturer's instructions. The PCR reaction mix consisted of 25 µl of a mixture containing: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each sequence-specific primer, 3 µl SYBR Green I (1:15 000), 3 µl of transcribed cDNA, and 0.5 U *Taq* polymerase (Biotools). The following PCR program was used: denaturation at 94 °C for 2 min; 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; and finally an extension step at 72 °C for 5 min. Supplementary Table S1 (available at *JXB* online) depicts the primer sequences used for quantitative amplification. Each reaction was performed, at least, in triplicate, and the corresponding C_t values were normalized using the C_t value corresponding to an interspacer 26S–18S strawberry RNA gene (Benítez-Burraco *et al.*, 2003; Raab *et al.*, 2006; Encinas-Villarejo *et al.*, 2009; Cumplido-Laso *et al.*, 2012). All these values were then used to determine the relative increase or decrease of *FaMYB10* expression in the samples as compared against those in control, in accordance with Pedersen (2001). Preliminary transcriptome analyses by microarrays of green strawberry fruits vs. red fruits, vegetative tissues, and de-achened fruits allowed a group of genes whose expression was constant in all these situations to be identified (unpublished data). The stable expression of these genes was afterwards corroborated by qRT-PCR (Supplementary Table S2, available at *JXB* online). When the expression of these genes was analysed by qRT-PCR and normalized between them, it was found that the *interspacer 26S–18S* was the housekeeping gene with less variability (data not shown). Thus, the *interspacer 26S–18S* (forward and reverse primers 5′-ACCGTTGATTCGCACAATTGGTCATCG-3′ and 5′-TACTGCGGGTCGGCAATCGGACG-3′) was selected as control for its constitutive expression throughout the different experimental conditions tested. The efficiency of each particular qRT-PCR and the melting curves of the products were also analysed to ensure the existence of a single amplification peak corresponding to a unique molecular species.

Generation of RNAi constructs and transfection of strawberry fruits by agroinfiltration

FaMYB10 was cloned into pFRN binary vector (Supplementary Fig. S1, available at *JXB* online; courtesy of Marten Denekamp from Department of Molecular Cell Biology, University of Utrecht, The Netherlands) using Gateway technology (Invitrogen, Darmstadt, Germany). A 407-bp conserved region from *FaMYB10* cv. Camarosa was amplified and used for generating RNAi fragments in the silencing construct. The forward primer 5′-AGATGACTAGATGATTGCTTGCCG-3′ and reverse primer 5′-TGCCGGACGATTGCCAGGAAG-3′ were used to that end. The resulting fragment was cloned into the pCR8/GW/TOPO (Invitrogen) and subsequently transferred to the Gateway pFRN vector by specific recombination between both plasmids by using LR clonase (Invitrogen). The RNAi construct-generated (pFRN-*FaMYB10*) was tested by sequencing and conducting restriction analyses prior to transformation of strawberry plants. The pFRN-*FaMYB10* construct generates RNAi directed against the endogenous *FaMYB10*.

Agrobacterium tumefaciens AGL0 (Lazo *et al.*, 1991) containing the pFRN-*FaMYB10* or empty pFRN vector was grown at 28 °C in fresh Luria–Bertani medium supplemented with appropriate antibiotics. When the culture reached an OD_{600} of about 0.8, cells were harvested and resuspended in a modified medium (Murashige and Skoog salts, 10 mM morpholine ethanesulphonic acid pH 5.6, 20 g l^{-1} sucrose, and 200 µM acetosyringone), in accordance with Spolaore *et al.* (2001). Both suspensions were evenly and independently injected with a sterile 1 ml hypodermic syringe into the base of the entire fruits (*Fragaria* × *ananassa* cv. Elsanta), while they remained attached to the plant for about 14 d after pollination (Hoffmann *et al.*, 2006). A total of 15–25 strawberry plants and 30–40 agroinjected fruits were inoculated and analysed, respectively.

The same protocol was followed to generate the *FaMYB1*-RNAi and *FaNCED1-RNAi* constructs and silencing *FaMYB1* and *FaNCED1* expression in strawberry fruits. The forward and reverse primers

used for *FaMYB1* were 5′-CTGCTGCCAGAAGACGGAG-3′ and 5′-AAAGAAGCCCCGGAGAGGAA-3′; while for *FaNCED1* were 5′-CGGTGCCGCCAGCTACGCAT-3′ and 5′-GTGGGGG CCGCCAGAGGGAT-3′.

In all cases, the silencing percentage was determined by comparing the amounts of *FaMYB10*, *FaMYB1*, or *FaNCED1* transcripts in pFRN-*FaMYB10*-, pFRN-*FaMYB1*-, and pFRN-*FaNCED1*-agroinjected fruits, respectively, against those observed in empty pFRN-vector agroinfiltrated fruits.

Phylogenetic analysis

The alignment of sequences and a phylogenetic tree presented in the supplementary data was obtained using the TCoffee suite of programs. Representations were obtained with the FigTree program.

Results

FaMYB10 *is a receptacle-specific gene expressed mainly in ripened and senescent fruit.*

By means of semi-quantitative real-time PCR (qRT-PCR), the level of FaMYB10 expression was analysed in both receptacles and achenes along different stages of fruit development and ripening. In receptacles, FaMYB10 was expressed at very low levels during the early G1, G3, and W stages of fruit development (Fig. 1A). A substantial and dramatic increase in its transcript levels took place, however, during the R, OR, and SN stages, with a peak of maximum expression observed in the OR stage.

In achenes, *FaMYB10* was expressed at very low levels throughout all developmental and ripening stages studied when compared against the expression level obtained in the fruit receptacle at the G1 stage (Fig. 1A). Negligible expression levels were observed in all vegetative tissues analysed, such as leaves, crowns, roots, and runners (Fig. 1B).

Taken together, all these data allowed *FaMYB10* to be defined as a receptacle-specific gene predominantly expressed during the ripening and senescent stages of fruit development.

Correlation of FaMYB10 *expression with anthocyanin content in strawberry receptacle tissues*

Anthocyanin accumulation in fruit receptacles is not uniform. These pigments are mainly accumulated in the external parenchyma, followed in amount by internal parenchyma, and with the lowest concentration of pigment accumulated in the pith. When the different parts of the receptacles were dissected and analysed, a clear and close relationship was observed in both anthocyanin content and level of FaMYB10 expression (Fig. 2).

Besides, *FaMYB10* reduction by transient silencing of its expression was also accompanied by a clear decrease in the anthocyanin content of the receptacles (Fig. 3). These results are in agreement with previous data showing an accumulation of anthocyanins in 35S overexpressed *FaMYB10* plants (Lin-Wang *et al.*, 2010) and support the involvement of *FaMYB10* in the anthocyanin production in strawberry fruits throughout the ripening process.

Transcriptomic analyses show that FaMYB10 *is a key regulator of the F/P pathway*

Transcriptome analysis was used to identify putative target genes affected by silencing of *FaMYB10*. A custom-made oligo-based microarray platform representing 34 789 putative genes from the *Fragaria* genome (FraGenomics35K) was used to compare gene expression profiles of transgenic transiently silenced *FaMYB10* fruits against control fruits agroinfiltrated with the empty pFRN vector (Supplementary Table S3, available at *JXB* online). The work focused on the most clearly downregulated genes by choosing a 2.0-fold cut off and a *P*-value ≤ 0.05. This microarray analysis revealed 30 transcripts that were downregulated in transiently *FaMYB10*-silenced fruits (Table 1). Out of them, 14 genes belong to genes encoding early and late biosynthetic enzymes of the F/P pathway, such as phenylalanine ammonia lyase (*PAL*; *GENE09753*), cinnamate-4-hydroxylase (*C4H*; *GENE28093*), flavanone 3-hydroxylase (*F3H*; *GENE14611*),

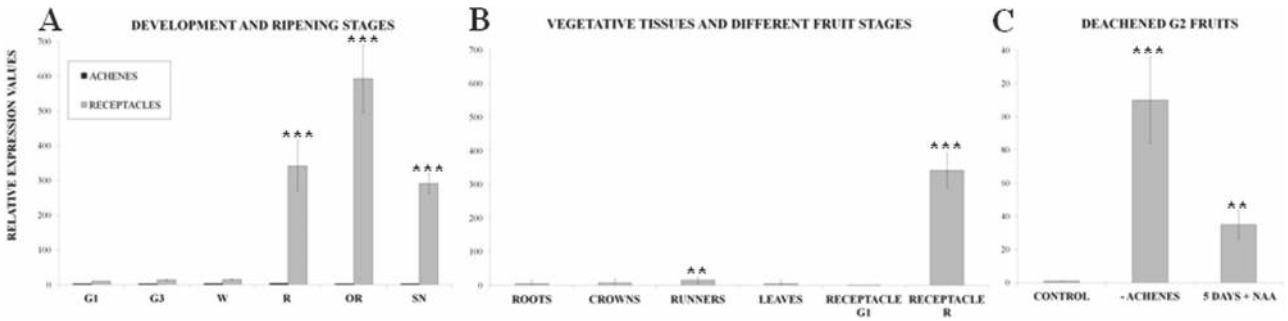

Fig. 1. Analysis by quantitative real-time PCR of *FaMYB10* expression in developing fruit receptacles and achenes (A), vegetative tissues (B), and de-achened G2 fruits (C) of *Fragaria* × *ananassa* cv. Camarosa: G1, small-sized green fruit; G3, full-sized green fruit (both stages of development); W, white stage; R, red stage; OR, overripe stage; SN, senescent stage; control, middle-sized green fruit receptacle (G2 fruit); –achenes, G2 fruit receptacle without achenes for 5 d; 5 days + NAA, G2 fruit receptacle without achenes plus 1-naphthaleneacetic acid for 5 d (added at day 0). Results were obtained using specific primers for *FaMYB10* and quantification is based on C_t values. In A, B, and C, relative expression is calculated relative to achenes' G1, G1-stage receptacle, and de-achened G2 fruits, respectively, which was assigned an arbitrary value equal to unity. Values are mean ± SD of five independent experiments. Statistical significance with respect to the reference sample was determined by the Student's t-test: $^{**}P < 0.01$; $^{***}P < 0.001$.

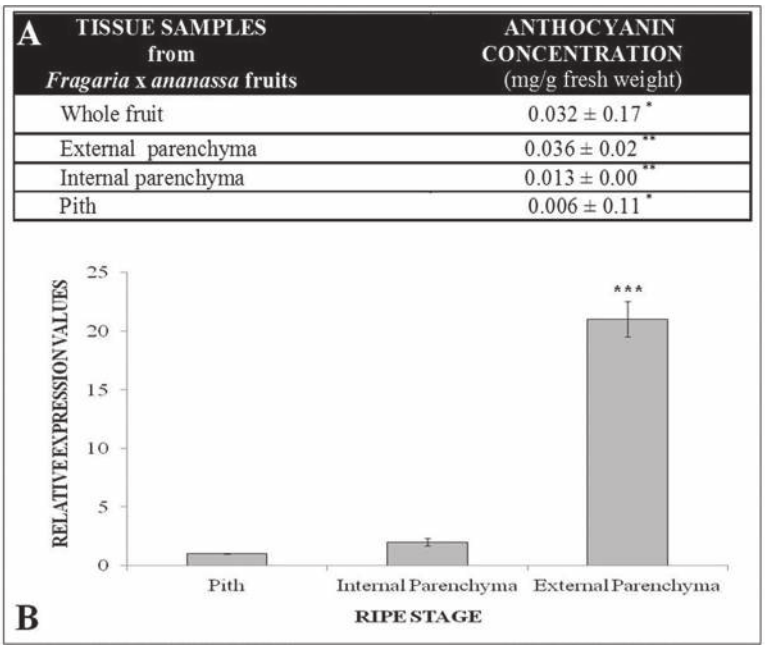

Fig. 2. (A) Quantification of anthocyanins in different red-stage *Fragaria* × *ananassa* fruit tissues; values are absorbance measurements at 515 nm of processed extracts from red fruits: $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Analysis by quantitative real-time PCR of *FaMYB10* expression in pith, internal, and external parenchyma tissues from red-stage Fragaria × ananassa fruits. relative expression is calculated relative to the pith C_t, which registered the lowest *FaMYB10* expression and was assigned an arbitrary value equal to unity. Values are mean ± SD of three independent experiments. Statistical significance with respect to reference sample was determined by the Student's t-test: $^{**}P < 0.01$; $^{***}P < 0.001$.

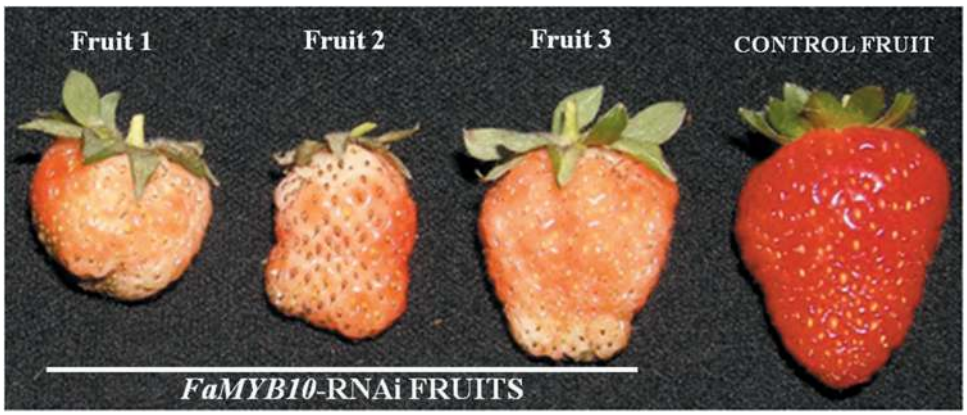

Fig. 3. Strawberry transgenic fruits (*Fragaria* × *ananassa* cv. Elsanta) agroinfiltrated with *FaMYB10*-pFRN construct. Control fruit, green–white fruit agroinfiltrated with the empty pFRN vector; *FaMYB10*-RNAi fruits, green–white fruits agroinfiltrated with *FaMYB10*-pFRN construct.

4-coumarate-CoA ligase (*4CL*; *GENE09603*), chalcone synthase (*CHS*; *GENE26826*), chalcone isomerase (*CHI*; *GENE21346*), dihydroflavonol reductase (*DFR*; *GENE29482*), UDP-glucose:flavonoid-3-*O*-glucosyltransferase (*UFGT*; *GENE12591*), cinnamoyl-CoA reductase (*GENE29483*), cinnamyl alcohol dehydrogenase (*CAD*; *GENE20700*), and two putative phenylcoumaran benzylic ether reductases 3 (*GENE25260* and *GENE25258*). Shikimate dehydrogenase (*GENE22235*), which is responsible for the synthesis of precursors of the F/P pathway, was also downregulated in silenced *FaMYB10*-RNAi transgenic fruits (Table 1).

In addition to the F/P pathway's structural genes, the expression of genes that are potentially involved in the transport of flavonoids, such as those showing strong similarity with MATE (*GENE15073*) or GSTs transporters (*GENE18167*), was also downregulated in transgenic *FaMYB10*-silenced fruits (Table 1).

It is noteworthy that the expression of R2R3-MYB (*FaEOBII*, *GENE28435*) ripening-related TFs was also downregulated when *FaMYB10* was silenced. This result strongly indicates that this TF acts downstream of *FaMYB10*.

Table 1. *Transcriptomic and qRT-PCR analyses of the main genes whose expression changes in transgenic* FaMYB10*-RNAi fruits, in fruits treated with NDGA, in de-achened fruits, and during fruit ripening*

NDGA and minus achenes indicate the expression obtained for the genes studied in green–white fruits injected with NDGA 100 μm and green2 fruits whose achenes were removed, respectively. Intensity indicates the total signal intensity for each feature on the microarray platform.

Orthologous gene in *Fragaria vesca*	Putative function	Species	E-value	Best match (BlastX)	Intensity in red fruit (AU)	*FaMYB10*-RNAi (down)			Red vs. green fruit (up)	NDGA (down) qRT-PCR	Minus achenes (up)
						Microarray		qRT-PCR			
						Fold	*P*-value				
Flavonoids											
GENE22235	Shikimate dehydrogenase	*Populus trichocarpa*	0.0	XM_002319546.1	4753.16	12.587	0.00696	7.24 ± 0.013	9.5 ± 0.99	9.23 ± 2.19	5.83 ± 0.88
GENE09753	Phenylalanine ammonia lyase (PAL)	*Rubus idaeus*	0.0	AF237955.1	19382.11	30.385	0.00099	44.65 ± 13.19	4.94 ± 0.29	27.58 ± 3.77	6.59 ± 1.18
GENE28093	Cinnamate-4-hydroxylase (C4H)	*Rubus occidentalis*	0.0	FJ554629.1	17109.34	12.028	0.00303	8.56 ± 1.09	6.91 ± 0.87	15.89 ± 2.07	5.17 ± 2.08
GENE14611	Flavanone 3-hydroxylase (F3H)	*Fragaria* × *ananassa*	0.0	AB201760.1	36215.99	111.839	0.00143	20.35 ± 3.12	6.75 ± 1.01	19.21 ± 1.94	10.28 ± 2.72
GENE09603	4-coumarate-CoA ligase (4CL)	*Arabidopsis thaliana*	0.0	NM_116755.4	19750.94	11.699	0.01690	7.68 ± 0.89	45.67 ± 16.21	21.69 ± 1.98	7.91 ± 2.76
GENE26826	Chalcone synthase (CHS)	*Fragaria* × *ananassa*	0.0	AY997297.1	29987.35	22.224	0.00099	10.45 ± 2.55	6.2 ± 0.78	14.76 ± 2.19	4.55 ± 0.71
GENE21346	Chalcone isomerase (CHI)	*Fragaria* × *ananassa*	5.00E-154	AB201755.1	12678.08	53.847	0.00132	50 ± 9.7	7.66 ± 2.23	20.35 ± 3.56	11.52 ± 0.92
GENE29482	Dihidroflavonol reductase (DFR)	*Vitis vinifera*	1.00E-146	XM_003633468.1	6231.56	15.476	0.00210	21.65 ± 2.27	36.32 ± 11.93	4.45 ± 0.85	21.05 ± 4.66
GENE12591	UDP glucose:flavonoid-3-*O*-glucosyltransferase (UFGT)	*Fragaria* × *ananassa*	0.0	AY575056.1	25165.04	75.901	0.00127	36.32 ± 7.71	1007 ± 267	19.67 ± 0.98	318.5 ± 15.79
GENE29483	Cinnamoyl-CoA reductase (CCR)	*Populus trichocarpa*	1.00E-158	XM_002314016.1	7357.27	3.861	0.0198	11.35 ± 1.087	45.79 ± 12.92	45.98 ± 4.77	16.32 ± 3.35
GENE20700	Cinnamyl alcohol dehydrogenase (CAD)	*Fragaria* × *ananassa*	0.0	U63534.1	9849.51	37.965	0.00108	12.97 ± 2.241	205 ± 17.79	88.21 ± 12.5	49.42 ± 9.43
GENE25260	Phenylcoumaran benzylic ether reductase 3 (PCBER3)	*Pyrus communis*	1.00E-170	AF071477.1	9021.06	8.580	0.000409	15.36 ± 1.31	15.67 ± 1.22	13.42 ± 2.41	32.74 ± 5.98
GENE25258	Phenylcoumaran benzylic ether reductase 3 (PCBER3)	*Populus trichocarpa*	4.00E-176	XM_002313752	4052.9	23.63	0.000015	55.65 ± 8.21	25.64 ± 2.57	3.546 ± 0.59	4.660 ± 1.001
Transporters											
GENE15073	MATE efflux family protein	*Arabidopsis thaliana*	0.0	NM_103646.3	3258.25	127.31	0.00289	157.87 ± 2.89	31.2 ± 3.22	23.45 ± 2.98	29.77 ± 2.27
GENE18167	Glutathione-S-transferase	*Rheum australe*	2.00E-114	EU931209.1	19749.03	9.72	0.00260	7.61 ± 1.97	6.77 ± 1.43	13.78 ± 2.09	22.47 ± 2.76
Transcription factors											
GENE31413	R2R3-MYB transcription factor (FaMYB10)	*Fragaria* × *ananassa*	2.00E-134	EU155162.1	12450.20	419.443	0.00111	623.09 ± 3.21	195.76 ± 22.7	14.46 ± 2.02	195.6 ± 23.19
GENE28435	Myb EOII	*Pisum sativum*	5.00E-82	Y11105.1	8801.50	3.58	0.00170	7.87 ± 1.77	148.9 ± 19.78	9.35 ± 0.85	7.31 ± 0.77
Other genes											
GENE21848	AAA-ATPase-like protein	*Solanum tuberosum*	0.0	DQ191627.1	560.83	7.93	0.00880	24.012 ± 5.23	31.78 ± 1.27	21.65 ± 3.49	14.41 ± 4.19
GENE22484	Cyclo-DOPA 5-*O*-glucosyltransferase	*Mirabilis jalapa*	2.00E-148	AB182643.1	10126.32	40.927	0.000013	112.9 ± 15.19	21.97 ± 2.89	7.21 ± 1.55	16.27 ± 2.55
GENE25718	Hexokinase 1	*Eriobotrya japonica*	0.0	JF414121.1	2347.71	3.054	0.00128	6.84 ± 1.17	6.23 ± 1.11	10.31 ± 1.12	5.89 ± 0.87

Table 1. Continued

Orthologous gene in *Fragaria vesca*	Putative function	Species	E-value	Best match (BlastX)	Intensity in red fruit (AU)	*FaMYB10*-RNAi (down)			Red vs. green fruit (up)	NDGA (down) qRT-PCR	Minus achenes (up)
						Microarray		qRT-PCR			
						Fold	*P*-value				
GENE11606	Sucrose-phosphate synthase 1 (putative)	*Prunus persica*	0.0	EF568781	1729.24	13.392	0.000265	32.45 ± 3.78	11.94 ± 2.03	6.328 ± 1.72	5.71 ± 0.98
GENE32435	Short-chain dehydrogenase/ reductase	*Nandina domestica*	3.00E-98	FJ789568.1	16553.98	10.539	0.00172	21.55 ± 3.98	13.77 ± 2.13	12.39 ± 2.46	40.59 ± 1.09
GENE31924	2-Oxoglutarate-dependent dioxygenase	*Populus trichocarpa*	2.00E-151	XM_002313047.1	4407.30	22.167	0.00160	46.32 ± 11.15	68.99 ± 9.12	9.62 ± 2.55	58.12 ± 12.45
GENE21312	Uncharacterized protein	*Gossypium hirsutum*	1.00E-21	EU373080.1	8787.08	5.03	0.00095	12.77 ± 0.97	9.63 ± 1.79	12.77 ± 1.19	19.37 ± 1.38
GENE30819	Uncharacterized protein	*Malus* × *domestica*	8.00E-24	CN489695	1738.81	236.561	0.00117	510.2 ± 24.75	118.9 ± 16.67	20.09 ± 3.16	11.29 ± 2.02
GENE11236	Uncharacterized protein	*Fragaria vesca*	2.00E-99	EX680210	1388.05	6.717	0.00136	13.96 ± 1.98	25.61 ± 2.05	19.22 ± 2.05	40.29 ± 10.02
GENE17017	Uncharacterized protein	*Fragaria vesca*	2.00E-131	DY673496.1	13569.57	75.438	0.00253	73.34 ± 11.13	72.56 ± 3.44	21.12 ± 4.72	39.66 ± 3.89
GENE10557	Uncharacterized protein	*Prunus avium*	1.00E-23	GAJZ01007755	6695.88	4.203	0.00352	10.11 ± 1.97	9.79 ± 1.97	6.476 ± 9.79	5.34 ± 0.92
GENE13154	Uncharacterized protein	*Fragaria vesca*	2E-66	XP_004308046.1	2819.79	4.581	0.0341	11.78 ± 1.99	12.99 ± 0.96	5.622 ± 0.91	4.41 ± 0.43

AU, arbitrary units; down, gene expression downregulated; NDGA, nordihydroguaiaretic acid; qRT-PCR, quantitative real-time PCR; up, gene expression upregulated.

A set of different genes, not F/P related, were also downregulated in silenced *FaMYB10*-RNAi transgenic fruits, such as those potentially coding an AAA-type ATPase (*GENE21848*), a cyclo-DOPA 5-*O*-glucosyltransferase (*GENE22484*), a hexokinase-1 (*GENE25718*), a putative sucrose-phosphate synthase 1 (*GENE11606*), a short-chain dehydrogenase/ reductase (*GENE32435*), and an 2-oxoglutarate-dependent dioxygenase (*GENE31924*). Further, the expression of a set of genes, still functionally uncharacterized, was downregulated (Table 1).

The expression of all these genes identified as downregulated in microarray analyses was confirmed using qRT-PCR. Additionally, the expression of these genes in NDGA-treated fruits as well as in unripe de-achened receptacles was also determined through this methodology. Interestingly, the expression of all these genes was also upregulated by both ripening and ABA receptacle content. On the contrary, the expression of these genes was downregulated by auxins released to the fruit receptacle from the achenes (Table 1).

Metabolite profiling of FaMYB10*-silenced receptacles confirms that this TF is a general regulator of F/P metabolism in ripened strawberry fruits*

To determine if the transcriptomic changes observed in FaMYB10-silenced fruits were accompanied by metabolite variations, the major soluble phenolic compounds were quantified by LC-MS in both control and transgenic *FaMYB10*-RNAi fruits (Fig. 4). As expected, substantially lower levels of anthocyanins (pelargonidin-3-glucoside, pelargonidin-3-glucoside-malonate, and cyanidin-3-glucoside) and flavonols (kaempferol-glucoside, kaempferol-glucuronide, and quercetin-glucuronide), but higher concentrations of phenylpropanoids (cinnamoyl glucose, caffeoyl glucose, and feruloyl glucose), were detected in transiently silenced fruits compared to untreated fruit. In contrast to this observation, the level of the phenylpropanoid 4-coumaroyl glucose decreased, and the concentration of the flavonoid naringenin-glucoside increased, in FaMYB10-RNAi fruits. The amount of catechin and epicatechin-catechin-dimers remained unchanged, while higher and lower levels of epiafzelechin-glucoside and epiafzelechin-catechin-dimers were detected in transgenic fruit, respectively. The concentrations of some metabolites not directly involved in the phenolics pathway (ascorbic acid and 4-hydroxy-2,5-dimethyl-3(2H)-furanyl glucoside (HDMF-glucoside)) were also affected by FaMYB10 silencing.

FaMYB1 TF is not a regulator of FaMYB10 *expression and vice versa*

The expression of a MYB TF from *Fragaria* × *ananassa* (*FaMYB1*) in tobacco flowers was reported to reduce the accumulation of some flavonoid compounds (Aharoni *et al.*, 2001). Recently, an orthologue of *FaMYB1* was isolated in the native white *Fragaria chiloensis* subsp. *chiloensis* (*FcMYB1*; Salvatierra *et al.*, 2013). The transitory silencing of *FcMYB1* expression renders fruits red. Additionally, the expression of some of the LBG flavonoid genes and of

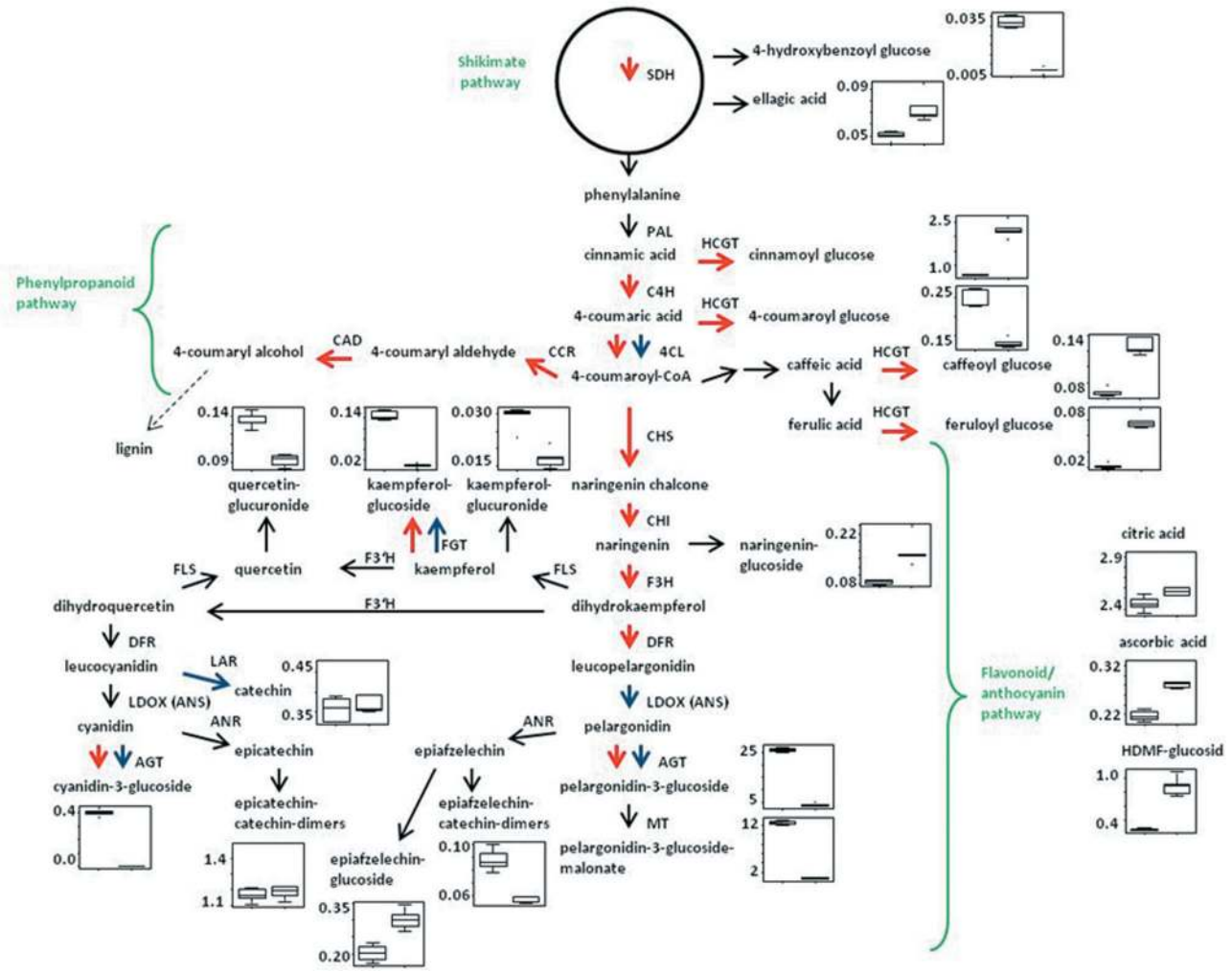

Fig. 4. Part of the phenylpropanoid, flavonoid, and anthocyanin pathway. Enzymes whose genes are down- and upregulated due to *FaMYB10* silencing are shown in red and blue, respectively. Boxplots show the levels of metabolites in control fruit (left) in comparison with their levels in FaMYB10-silenced fruit (right). 4CL, 4-coumaroyl-CoA ligase; AGT, anthocyanidin glucosyltransferase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; C4H, cinnamic acid 4-hydroxylase; CAD, 4-coumaryl alcohol dehydrogenase; CCR, 4-coumaroyl-CoA reductase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FGT, flavonoid glucosyltransferase; HCGT, (hydroxy)cinnamic acid glucosyltransferase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; MT, malonyl transferase; PAL, phenylalanine ammonia lyase; SDH, shikimate dehydrogenase.

pelargonidin-3-glucoside content was also slightly increased (Salvatierra *et al.*, 2013).

In order to determine if *FaMYB10* is under the regulation of FaMYB1, this work transiently suppressed *FaMYB1* expression in *Fragaria* × *ananassa* cv. Elsanta. Despite obtaining a high percentage of gene silencing in *FaMYB1*-silenced receptacles, no changes in *FaMYB10* expression were observed (Fig. 5A). Similarly, *FaMYB1* expression remained unaltered in fruit receptacles where *FaMYB10* expression was suppressed. Anthocyanin content of *FaMYB1*-silenced fruit receptacles remained unchanged with regard to those observed in control fruits (Fig. 5B and C). These results rule out a direct regulatory role of FaMYB1 on *FaMYB10* expression and vice versa and indicate that FaMYB1 does not play a similar role to that of FcMYB1 in *F. chiloensis*.

FaMYB10 *expression in fruit receptacles is repressed by auxin and activated by ABA*

It has been proposed that a defined ABA/auxin content ratio present in receptacles could be a signal that triggers the fruit-ripening process (Perkins-Veazie, 1995; Jiang and Joyce, 2003). Auxins are mainly produced by the achenes and released afterwards to the receptacles, fostering receptacle growth and development while preventing premature ripening. Some recent reports have suggested that the phytohormone ABA may, at least, be involved in anthocyanin production during the ripening of strawberry fruits (Chai *et al.*, 2011; Jia *et al.*, 2011). The current work assumed that these two hormones affect FaMYB10 expression.

To analyse the in vivo effects of auxins, two complementary experiments were conducted. The first mechanically removed

the achenes, and thus the source of auxins from several G2 fruits, and compared *FaMYB10* transcript levels through qRT-PCR after 5 d with those of untreated control fruits. A clear and substantial increase in the amount of FaMYB10 transcripts was observed in de-achened unripe fruit receptacles in comparison to control fruits. The second experiment determined that the increase of FaMYB10 expression in de-achened fruits was abolished by the external application of auxin (1-naphthaleneacetic acid; Fig. 1C).

To assess the role played by ABA in the regulation of *FaMYB10* expression, this work modulated ABA content (down and up) through three experimental approaches: (1) transiently silencing the expression of *FaNCED* coding 9-*cis*-epoxycarotenoid dioxygenase, the key enzyme responsible for ABA biosynthesis through *in vivo* agroinfiltration with live *Agrobacterium* cells harbouring *FaNCED1*-RNAi constructs; (2) adding NDGA, a well-known inhibitor of 9-*cis*-epoxycarotenoid dioxygenase activity; and (3) depleting plants of

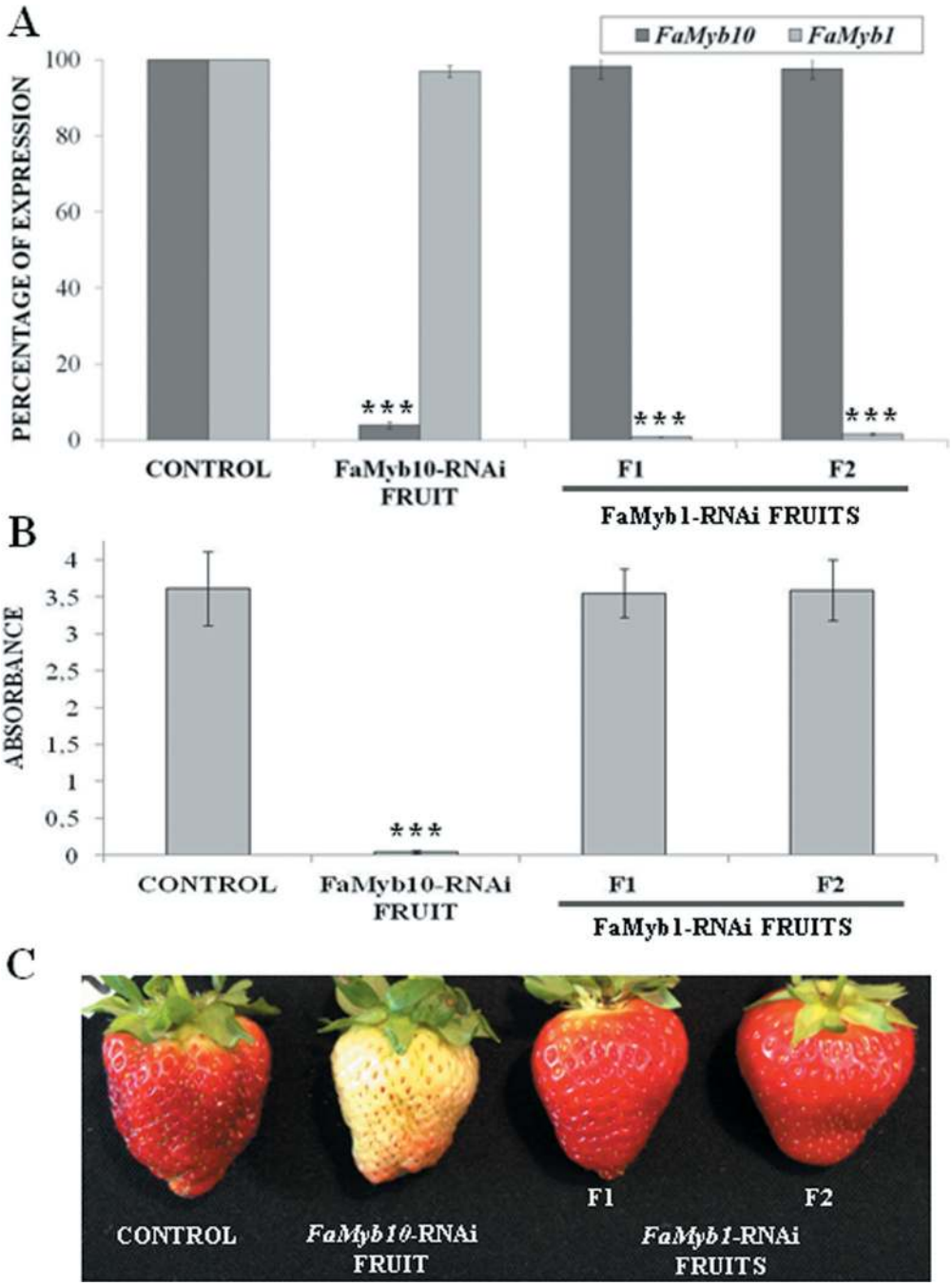

Fig. 5. (A) *FaMYB10* and *FaMYB1* expression in transgenic agroinfiltrated fruits injected with the corresponding RNAi contsruct. (B) Determination of anthocyanin content in transgenic *FaMYB10*-RNAi and *FaMYB1*-RNAi fruits. (C) Phenotypes of transgenic *FaMYB10*-RNAi and *FaMYB1*-RNAi fruits. Statistical significance with respect to control sample was determined by the Student's t-test: *** $P < 0.001$ (n = 10).

water, as water stress is known to increase ABA content in the plants. All of these experiments were accompanied by the measurement of ABA content in fruit receptacles.

A lack of red colouration was observed in fruits that were either agroinfiltrated with *FaNCED1*-RNAi constructs or treated with the inhibitor NDGA (Fig. 6A and B). In contrast, an early appearance as well as a substantial increase of fruit red colouration was noted in fruits subjected to water stress (Fig. 6C). It is noteworthy that agroinfiltration was conducted with *Agrobacterium* harbouring the *FaNCED1*-RNAi construct in half of the receptacles, while the other half was agroinfiltrated with bacteria harbouring the same vector but lacking the RNAi sequences that were thus used as control. No noticeable changes were observed in the half lacking the RNAi construct in relation to untreated fruits.

Substantial changes in ABA content (either increase or decrease) in receptacles occurred under the three different experimental procedures described above (Fig. 7B, D, F). In all of these cases, *FaMYB10* expression always correlated with that of ABA content (Fig. 7A, C, E). These results strongly suggest that ABA presence in the receptacles can positively regulate the expression of FaMYB10 and that this TF plays an important role in the regulation of genes involved in the anthocyanin biosynthesis in this fruit.

Taken together, these results indicate that *FaMYB10* expression is clearly regulated by both ABA content and auxin content of the fruit receptacle, which provides a putative molecular explanation for both the proposal of Perkins-Veazie (1995) and the findings described by Chai *et al.* (2011) and Jia *et al.* (2011).

Discussion

This paper presents data indicating that the *Fragaría* × *ananassa* cv. Camarosa gene *FaMYB10* encodes a TF that plays a key role in the regulation of the F/P pathway in fruit receptacle during the ripening of strawberry fruit.

Expression of FaMYB10 *is fruit specific and related to the expression of genes involved in F/P metabolism*

FaMYB10 expression was confined mainly to the fruit receptacles, not being or barely being expressed in all other vegetative tissues analysed such as leaves, stems, and runners as well as in fruit achenes. The expression was ripening related insomuch as its expression was also very low in the early development stage of the fruit but it experienced a substantial increase during the ripening stages, and it coincided with the development of the red colour in the fruit when F/P metabolism was fully active. All these data taken together allow this gene to be defined as a truly and specific receptacle-specific gene that is related to ripening.

High-throughput transcriptomic analysis indicates that FaMYB10 plays a key role in the early, late, and transport steps of phenylpropanoids and flavonoids biosynthesis

The transcriptomic analysis presented herein revealed that many of the genes involved in F/P metabolism, from *PAL* to *UFGT*, can be directly or indirectly upregulated by FaMYB10. Interestingly, as in the case of *FaMYB10* expression, the

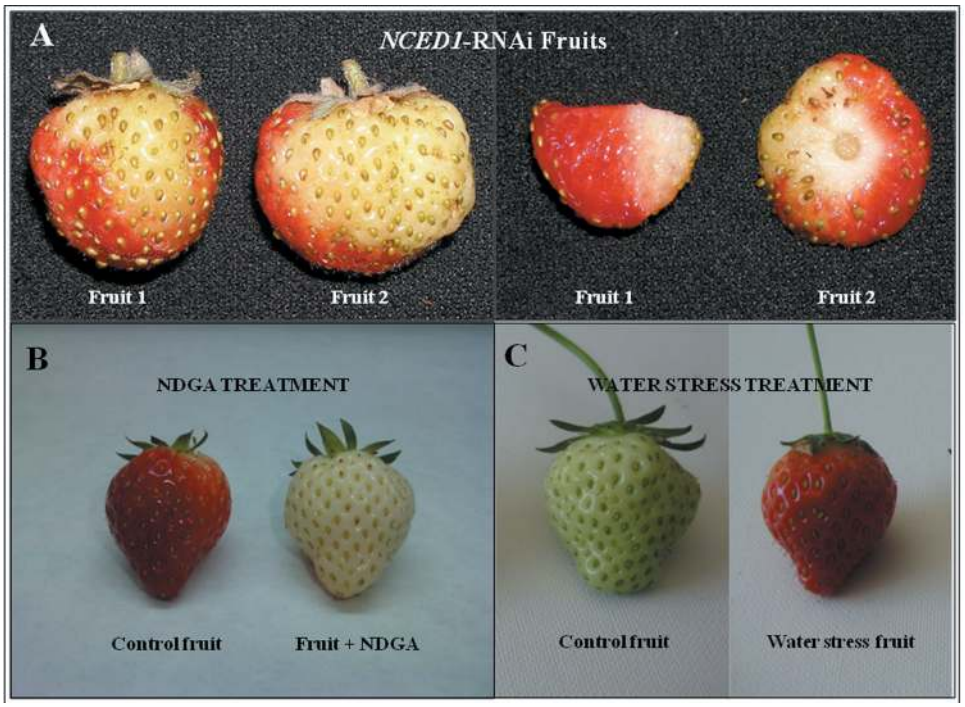

Fig. 6. Strawberry green–white fruits (*Fragaria* × *ananassa* cv. Elsanta) agroinfiltrated with the *NCED1*-RNAi construct (A), 8 d after treatment with 100 μM nordihydroguaiaretic acid (B; NDGA; control fruit, injected with H_2O), and 4 d after the beginning of water stress by keeping pedicels in the air (C; control fruit, pedicels immersed in MS medium with sucrose).

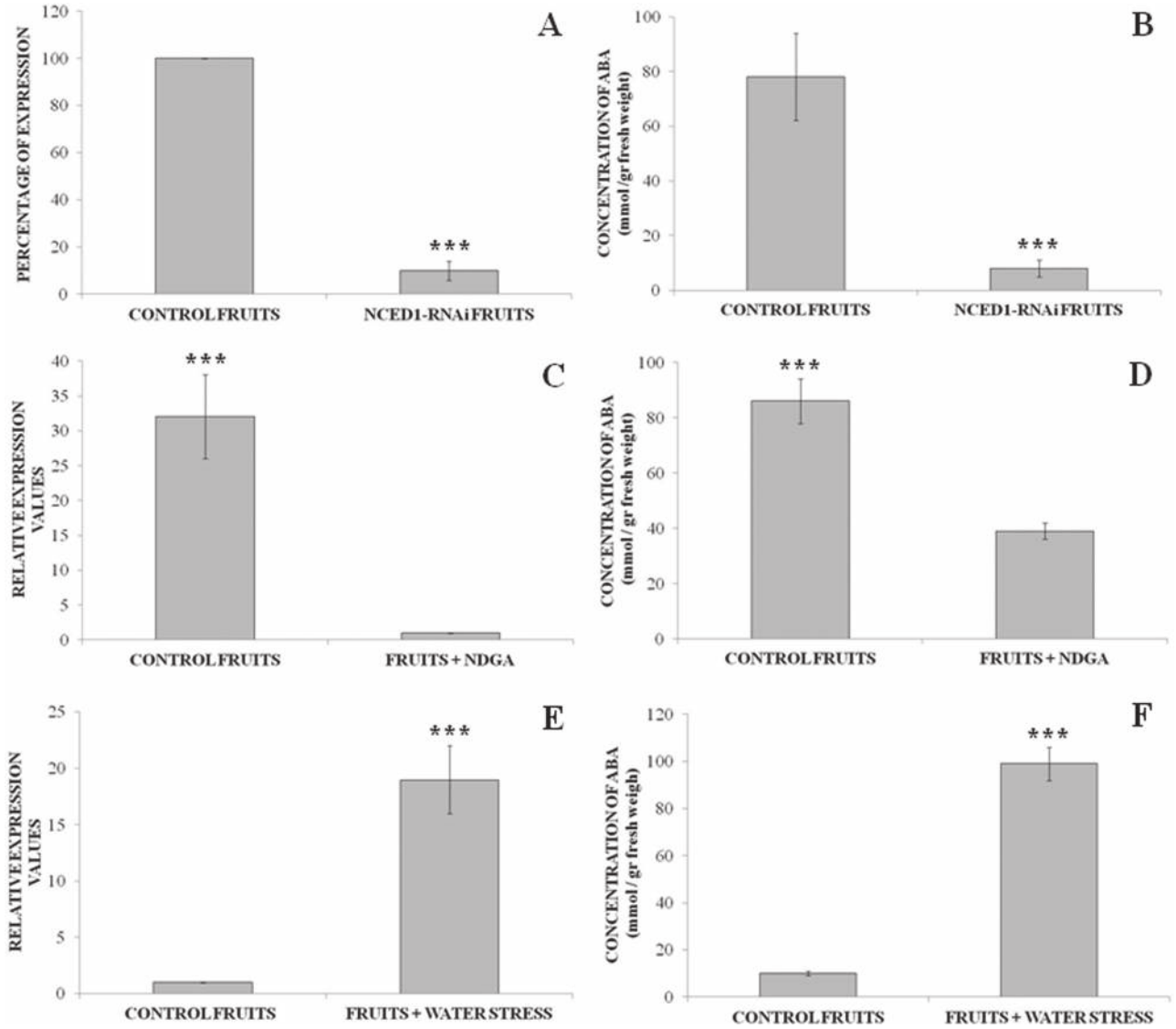

Fig. 7. Analysis by quantitative real-time PCR of *FaMYB10* expression (A, C, E) and quantification of ABA concentration (B, D, F): (A, B) control fruits, infiltrated with empty pFRN vector; NCED1-RNAi fruits, transgenic agroinfiltrated with the *FaNCED1*-pFRN construct; (C, D) control fruits, green–white fruit injected with H_2O; fruits + NDGA, green–white fruits injected with 100 μM nordihydroguaiaretic acid (NDGA); (E, F) control fruits, pedicels immersed in MS medium with sucrose; fruits + water stress, pedicels kept in the air. Statistical significance with respect to reference sample was determined by the Student's t-test; ***$P < 0.001$.

expression of all of these genes was also upregulated in the red ripened receptacles. This supports the possibility that FaMYB10 is fulfilling a key central role by regulating most of the biosynthetic genes related to the F/P pathway that are connected to ripening. An exception, however, is the expression of anthocyanidin synthase (*ANS*), which remained unchanged in *FaMYB10*-silenced receptacles, suggesting the existence of a different regulatory mechanism controlling the expression of this particular gene.

In *V. vinifera*, a similar broad regulatory role was demonstrated for VvMYB5b, a R2R3 TF that regulates all branches of the biosynthesis of flavonoids, including the anthocyanin and the phenylpropanoid pathways (Deluc *et al.*, 2008). However, VvMYB5b does not regulate *UFGT* expression, and this role is played by both VvMYBBA1 and VvMYBBA2 (Walker *et al.*, 2007). In strawberry, however, FaMYB10 strongly regulates the expression of strawberry *UFGT* as in pear, where the methylation level of *PcMYB10* promoter directly affects *PcUFGT* expression (Wang *et al.*, 2013). These results suggest that FaMYB10 could play a broader regulatory role than that played by VvMYB5b, VvMYBA1, or VvMYBA2. In strawberry ripened receptacles, FaMYB10 apparently seems to regulate by itself the expression of all the genes regulated by these three MYB TFs in *V. vinifera.* As with *FaMYB10*, the overexpression of *MYB10* in pear and *Gerbera hybrida* (*GMYB10*) increased the expression of *PAL*, *C4H*, *CHI*, *F3H*, and *GST* in fruit peel (Espley *et al.*, 2013) and petals (Laitinen *et al.*, 2008), respectively. Interestingly, both VvMYB5b and GMYB10 regulate the expression of *ANS* as well, whereas FaMYB10 does not. However, as in

the case of *FaMYB10*, overexpression of *VvMYB5a* in stamens of grapevine induced all of the structural genes of the F/P pathway except *ANS*, and this activation correlated with a strong accumulation of anthocyanins in epidermal cells (Deluc *et al.*, 2006). It has been proposed that in strawberry, another MYB transcription regulator (FaMYB5) could influence the expression of *FaANS*, whose expression is not regulated by FaMYB10 (Schaart *et al.*, 2013). However, the expression of *FaMYB5* was not clearly induced through the receptacle-ripening stages. Besides, a putative negative regulatory role was suggested for this TF. In this sense, the potential regulatory role of FaMYB5 on *FaANS* expression must be still determined.

In strawberry, the R2R3-MYB1 TF is weakly induced in ripened fruits (Aharoni *et al.*, 2001), and it has been proposed to be a transcription repressor in the regulation of the production of anthocyanins in *Fragaria* × *ananassa* ripe fruit (Aharoni *et al.*, 2001). However, it has been shown that the ectopic overexpression of *FaMYB1* in tobacco flowers did not affect the expression of *PAL*, *C4H*, *4CL*, *CHS*, *F3H*, *DFR*, *UDP*-rhamnosyl transferase, and *CAD* (Aharoni *et al.*, 2001). In these same transgenic *FaMYB1* tobacco plants, the expression of *ANS* was clearly downregulated. On the contrary, transcriptomic analyses showed that all of these genes but *ANS* could be regulated by FaMYB10 in ripening fruits. Recently, the white Chilean strawberry phenotype (*F. chiloensis* subsp. *chiloensis* f. chiloensis) was reverted to a partially red phenotype through the silencing of the expression of *FcMYB1* (Salvatierra *et al.*, 2013). Notwithstanding this, the transcriptomic data did not rule out the possibility that FaMYB1 could influence the expression of those genes regulated by FaMYB10. This left open the possibility that, to some extent, FaMYB1 could directly regulate the expression of any of the F/P genes through the modulation of *FaMYB10* expression. However, the experimental data, obtained after transitory silencing of *FaMYB1* expression in fruit receptacle, strongly ruled out this possibility.

FaMYB10 also regulated the expression of *GST* and *MATE* transporters. In plants, GSTs and MATE proteins are required for vacuolar sequestration of flavonoids and anthocyanins (Mano *et al.*, 2007; Cutanda-Pérez *et al.*, 2009; Gomez *et al.*, 2011). Thus, a possibility is that both proteins could be associated together in ripening fruits in order to transport and store anthocyanins into the vacuole in strawberry fruit receptacle.

The transitory silencing of *FaMYB10* reduced levels of anthocyanins and flavonols that were detected in *FaMYB10*-RNAi fruits in comparison to control fruits. The concentrations of the proanthocyanins catechin and its dimer, however, were not altered (Fig. 4). On the other hand, the impact of *FaMYB10* downregulation on the amounts of epiafzelchin derivates was mixed. The higher levels of phenylpropanoyl glucosides in *FaMYB10*-RNAi receptacles point to a redirection of the pathway towards early intermediates of the phenolic pathway similar to that observed in *FaCHS*-RNAi fruit (Hoffmann *et al.*, 2006). Besides, two metabolites that are not directly related to the anthocyanin pathway, namely ascorbic acid and HDMF-glucoside, accumulated upon *FaMYB10* downregulation. These compounds are linked to carbohydrate metabolism and suggest alterations in this pathway that are consistent with the reduced transcript levels of the hexokinase-1 and sucrose phosphate synthase 1 genes (Raab *et al.*, 2006).

During the fruit-ripening process, of 146 genes encoding MYB TFs in the *F. vesca* genome (manually annotated), only 13 of them were induced in red-ripened receptacles (Supplementary Fig. S2 and Supplementary Table S4, available at *JXB* online). Among them, only *FaEOBII* expression was downregulated in *FaMYB10*-RNAi transgenic fruits, which suggests that this TF can be under the control of FaMYB10. *FaEOBII* shows a high sequence similarity with that of *EOBII* (Emission Of Benzenoids II) of *Petunia hybrida* that encodes a R2R3-MYB TF. This TF has been previously characterized as a regulator of structural phenylpropanoid genes that are involved in the production of the volatile phenylpropanoids components of the floral scent, such as phenylethyl alcohol, benzylbenzoate, eugenol, and isoeugenol (Spitzer-Rimon *et al.*, 2010). In this sense, the current work also observed that the expression of genes belonging to the phenylpropanoid pathway potentially involved in producing these volatiles, such as cinnamyl-CoA reductase, cinnamyl alcohol dehydrogenase, and a putative phenylcoumaran benzylic ether reductase 3, were also downregulated in transgenic *FaMYB10*-silenced fruits, probably through the downregulation of *FaEOBII* expression. The involvement of *EOBII* in the regulation of biosynthetic genes responsible for the production of phenylpropanoid volatiles is also supported by the high expression of both TFs in petals, where these volatiles are strongly produced and emitted (Spitzer-Rimon *et al.*, 2010).

Among the other genes whose expression was downregulated in *FaMYB10*-silenced receptacles, this work identified genes such as a receptor-like protein kinases, a AAA-type ATPases, a cyclo-DOPA 5-*O*-glucosyltransferase, a short-chain dehydrogenase/reductase, a putative sucrose-phosphate synthase 1, a hexokinase, and a 2-oxoglutarate-dependent dioxygenase. The precise functions of all these genes are currently unknown. However, it is noteworthy that similar genes are also regulated by the R2R3-MYB TFs GMYB10 and VlMYBA1, both of which are involved in anthocyanin production in *G. hybrida* and *Vitis labruscana*, respectively (Laitinen *et al.*, 2008; Cutanda-Pérez *et al.*, 2009).

Both FaMYB10 *expression, such as those genes regulated by this TF, are under the control of auxin and ABA*

Although orthologues of *MYB10* have been isolated from at least 20 rosaceous species (Lin-Wang *et al.*, 2010), little is known about the hormonal regulation of *FaMYB10*. In strawberry, it is generally assumed that auxins synthesized by the achenes and released to the receptacles in the early phase of fruit development foster fruit growth. The auxin content of receptacles decays while the receptacle size reaches its maximum volume (in the white stage). Afterwards, once the receptacle attains its final size (in the white stage), auxin production declines while ABA production increases inside the

receptacle, triggering the ripening process (Perkins-Veazie, 1995). According to this, the expression of a vast majority of strawberry ripening-related genes is being repressed by auxins (Medina-Escobar *et al.*, 1997a, b; Moyano *et al.*, 1998; Blanco-Portales *et al.*, 2002; Benitez-Burraco *et al.*, 2003; Raab *et al.*, 2006; Griesser *et al.*, 2008). However, the positive regulation of the expression corresponding to these ripening-related genes remains to be elucidated.

This work demonstrated that *FaMYB10* expression is also directly or indirectly downregulated by auxins. In fact, a strong increase of *FaMYB10* transcripts was observed when achenes were removed from the fruit at early stages of development. This increase was the consequence of a decrease of the auxin content in the receptacle (Medina-Escobar *et al.*, 1997a, b; Moyano *et al.*, 1998; Blanco-Portales *et al.*, 2002; Benitez-Burraco *et al.*, 2003; Raab *et al.*, 2006; Griesser *et al.*, 2008; Cumplido-Laso *et al.*, 2012; Molina-Hidalgo *et al.*, 2013). On the contrary, in de-achened unripe green fruits, an increase of the auxin content lead to a dramatic reduction in *FaMYB10* transcript level. This hormonal expression pattern of *FaMYB10* was similar to that observed for all genes regulated by this gene. These results were also coincident with those of many other functionally characterized ripening-related genes involved in the biosynthesis of compounds related to fruit organoleptic properties (Medina-Escobar *et al.*, 1997a, b; Moyano *et al.*, 1998; Blanco-Portales *et al.*, 2002; Benitez-Burraco *et al.*, 2003; Raab *et al.*, 2006; Griesser *et al.*, 2008; Cumplido-Laso *et al.*, 2012; Molina-Hidalgo *et al.*, 2013).

Some studies have indicated that once the receptacle reaches the ripening white stage, ABA promotes the strawberry ripening-related production of anthocyanins (Chai *et al.*, 2011; Jia *et al.*, 2011) and is likely to trigger the ripening process. However, although it is known that the level of ABA content gradually increases during ripening, little is known about how this hormone regulates strawberry ripening-related genes. In this sense, this work clearly shows that when the level of ABA in the receptacles was modulated, a concomitant and parallel change in the expression of *FaMYB10* took place. Thus, when ABA content in the receptacle decreased as a consequence of either the transient silencing of *FaNCED1* expression or by the inhibition of the corresponding enzyme activity by the inhibitor NDGA, a decrease in *FaMYB10* expression was observed (Fig. 7). Higher levels of the same transcript were observed, however, when ABA content increased as a consequence of subjecting the fruits to water stress. In fact, in both climacteric and non-climacteric fruits, water deficiency generated a raise in ABA content, which accelerated ripening and was accompanied by changes in both metabolites and gene expression (Castellarin *et al.*, 2007; Gong *et al.*, 2010). As in strawberry fruits and grapes, drought stress induces an increase in ABA content and, as consequence, an increase in the biosynthesis of flavonoids and the activation of drought-inducible genes (Yamaguchi-Shinozaki and Shinozaki, 2005). This work proved that changes in ABA content (lower or higher production) of the fruit receptacle were accompanied by parallel changes in the level of *FaMYB10* transcripts and of those of anthocyanins. Additionally, similar changes in gene expression were observed for all the genes transcriptionally regulated by *FaMYB10* as a result of transient *FaMyb10* silencing. These molecular results account for the recent results obtained by Chai *et al.* (2011) and Jia *et al.* (2011), who also observed a decrease in the anthocyanin content in ripened strawberry fruits when ABA production was blocked in receptacles. Besides, these results strongly support a relationship between the disappearance of auxins, the appearance of ABA, and the receptacle fruit growth and ripening, as proposed by Perkins-Veazie (1995).

In summary, this work presents novel functional data indicating that FaMYB10 is a receptacle-specific ripening-related TF that regulates *EBG* and *LBG* of the F/P pathway that are related to the biosynthesis of anthocyanins in the strawberry fruit receptacle. *FaMYB10* expression is strongly regulated by those hormonal clues related to strawberry fruit ripening, the expression of this gene being almost restricted to the fruit receptacle. The high-throughput transcriptomic analyses performed in transgenic strawberry fruits also show that FaMYB10 regulates the expression of other TFs putatively involved in ripening. All these data strongly indicate that FaMYB10 is a TF playing a general regulatory role during the ripening process in strawberry, mainly related to anthocyanin biosynthesis, which is very likely to play a prominent part in the signalling transduction cascade.

Supplementary material

Supplementary data are available at *JXB* online.
Supplementary Table S1. Primer sequences used for qRT-PCR.
Supplementary Table S2. Gene expression values of different housekeeping genes obtained by qRT-PCR analysis.
Supplementary Table S3. Transcriptomic analysis of transgenic transiently silenced *FaMYB10* fruits against control fruits agroinfiltrated with the empty pFRN vector.
Supplementary Table S4. Global transcriptomic comparison of MYB transcription factors in red ripened receptacles vs. green ripened receptacles.
Supplementary Fig. S1. Structure of binary vector pFRN.
Supplementary Fig. S2. Phylogenetic tree of MYB transcription factor analysed from *Fragaria vesca* genome in the global transcription study of red ripened receptacles vs. green unripe receptacles.

Acknowledgements

This work was supported by a grant from the Ministerio Español de Ciencia e Innovación (EUI2008-03668 and BIO2010-19322). LMP and GCL are grateful for the award of a PhD fellowship within the framework of FPU programme implemented by the Ministerio de Educación y Ciencia and a fellowship from the Comunidad Autonóma de Andalucía, respectively. RBP is grateful for the award of a post-doctoral contract within the framework of the Juan de la Cierva programme implemented by the Ministerio Español de Ciencia e Innovación. LR and WS thank BMBF for financial support in the framework of the FraGenomics project. The authors would also like to thank Nicolás García-Caparrós for his technical assistance in laboratory experiments.

References

Aharoni A, De Vos CHR, Wein M, Sun ZK, Greco R, Kroon A, Mol JNM, O'Connell AP. 2001. The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. *The Plant Journal* **28,** 319–332.

Asif MH, Dhawan P, Nath P. 2000. A simple procedure for the isolation of high quality RNA from ripening banana fruit. *Plant Molecular Biology Reporter* **18,** 109–115.

Ban Y, Honda C, Hatsuyama Y, Igarashi M, Bessho H, Moriguchi T. 2007. Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. *The Plant Cell and Physiology* **48,** 958–970.

Benitez-Burraco A, Blanco-Portales R, Redondo-Nevado J, Bellido ML, Moyano E, Caballero JL, Muñoz-Blanco J. 2003. Cloning and characterization of two ripening-related strawberry (*Fragaria* × *ananassa* cv. Chandler) pectate lyase genes. *Journal of Experimental Botany* **54,** 633–645.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society B* **57,** 289–300.

Blanco-Portales R, Medina-Escobar N, López-Ráez JA, González-Reyes JA, Villalba JM, Moyano E, Caballero JL, Muñoz-Blanco J. 2002. Cloning, expression and immunolocalization pattern of a cinnamyl alcohol dehydrogenase gene from strawberry (*Fragaria* × *ananassa* cv. Chandler). *Journal of Experimental Botany* **53,** 1723–1734.

Bombarely A, Merchante C, Csukasi C, *et al.*, 2010. Generation and analysis of ESTs from strawberry (*Fragaria* × *ananassa*) fruits and evaluation of their utility in genetic and molecular studies. *BMC Genomics* **11,** 503.

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*. *Theoretical and Applied Genetics* **109,** 23–29.

Bustamante CA, Civello PM, Martínez GA. 2009. Cloning of the promoter region of b-xylosidase (FaXyl1) gene and effect of plant growth regulators on the expression of FaXyl1 in strawberry fruit. *Plant Science* **177,** 49–56.

Carvalho RF, Quecini V, Pereira-Peres LE. 2010. Hormonal modulation of photomorphogenesis-controlled anthocyanin accumulation in tomato (*Solanum lycopersicum* L. cv Micro-Tom) hypocotyls: physiological and genetic studies. *Plant Science* **178,** 258–264.

Castellarin SD, Matthews MA, Di Gaspero G, Gambetta GA. 2007. Water deficits accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis in grape berries. *Planta* **227,** 101–112.

Chagne D, Carlisle CM, Blond C, *et al.* 2007. Mapping a candidate gene (*MdMYB10*) for red flesh and foliage colour in apple. *BMC Genomics* **8,** 212.

Chai Y-m, Jia H-f, Li C-l, Dong Q-h, Shen Y-Y. 2011. *FaPYR1* is involved in strawberry fruit ripening. *Journal of Experimental Botany* **62,** 5079–5089.

Chiu L-W, Zhou X, Burke S, Wu X, Prior RL, Li L. 2010. The purple cauliflower arises from activation of a MYB transcription factor. *Plant Physiology* **154,** 1470–1480.

Creelman RA, Bell E, Mullet JE. 1992. Involvement of a lipoxygenase-like enzyme in abscisic acid biosynthesis. *Plant Physiology* **99,** 1258–1260.

Cumplido-Laso G, Medina-Puche L, Moyano E, Hoffmann T, Sinz Q, Ring L, Studart-Wittkowski C, Schwab W, Muñoz-Blanco J, Blanco-Portales R. 2012. The fruit ripening-related gene *FaAAT2* encodes an acyl transferase involved in strawberry aroma biogenesis. *Journal of Experimental Botany* **63,** 4275–4290.

Cutanda-Perez M-C, Ageorges A, Gomez C, Vialet S, Terrier N, Romieu C, Torregrosa L. 2009. Ectopic expression of *VlmybA1* in grapevine activates a narrow set of genes involved in anthocyanin synthesis and transport. *Plant Molecular Biology* **69,** 633–648.

Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, Carde JP, Merillon JM, Hamdi S. 2006. Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. *Plant Physiology* **140,** 499–511.

Deluc L, Bogs J, Walker AR, Ferrier T, Decendit A, Merillon J-M, Robinson S.P, Barrieu F. 2008. The transcription factor VvMYB5b contributes to the regulation of anthocyanin and proanthocyanidin biosynthesis in developing grape berries. *Plant Physiology* **147,** 2041–2053.

Encinas-Villarejo S, Maldonado AM, Amil-Ruiz F, de los Santos B, Romero F, Pliego-Alfaro F, Muñoz-Blanco J, Caballero JL. 2009. Evidence for a positive regulatory role of strawberry (*Fragaria* × *ananassa*) *FaWRKY1* and *Arabidopsis* At WRKY75 proteins in resistance. *Journal of Experimental Botany* **60,** 3043–3065.

Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC. 2007. Red coloration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *The Plant Journal* **49,** 414–427.

Espley RV, Bovy A, Bava C, *et al.* 2013. Analysis of genetically modified red-fleshed apples reveals effects on growth and consumer attributes. *Plant Biotechnology Journal* **11,** 408–419.

Feild TS, Lee DW, Holbrook NM. 2001. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. *Plant Physiology* **127,** 566–574.

Gomez C, Conejero G, Torregrosa L, Cheynier V, Terrier N, Ageorges A. 2011. *In vivo* grapevine anthocyanin transport involves vesicle-mediated trafficking and the contribution of anthoMATE transporters and GST. *The Plant Journal* **67,** 960–970.

Gong P, Zhang J, Li H, Yang C, *et al.*, 2010. Transcriptional profiles of drought-responsive genes in modulating transcription signal transduction, and biochemical pathways in tomato. *Journal of Experimental Botany* **61,** 3563–3575.

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

Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V. 2011. Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *Journal of Experimental Botany* **62,** 2465–2483.

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

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

Jiang YM, Joyce DC. 2003. ABA effects on ethylene production, PAL activity, anthocyanin and phenolic contents of strawberry fruit. *Plant Growth Regulation* **39,** 171–174.

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

Laitinen RAE, Ainasoja M, Broholm SK, Teeri TH, Elomaa P. 2008. Identification of target genes for a MYB-type anthocyanin regulator in *Gerbera hybrida*. *Journal of Experimental Botany* **59,** 3691–3703.

Lazo GR, Pascal AS, Ludwig RA. 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology* **9,** 963–967.

Lin-Wang K, Bolitho K, Grafton K, Kortstee A, Karunairetnam S, McGhie TK, Espley RV, Hellens RP, Allan AC. 2010. An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in *Rosaceae*. *BMC Plant Biology* **10,** 50.

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

Medina-Escobar N, Cárdenas J, Moyano E, Caballero JL, Muñoz-Blanco J. 1997a. Cloning, molecular characterization and expression pattern of a strawberry ripening-specific cDNA with sequence homology to pectate lyase from higher plants. *Plant Molecular Biology* **34,** 867–877.

Medina-Escobar N, Cárdenas J, Valpuesta V, Muñoz-Blanco J, Caballero JL. 1997b. Cloning and characterization of cDNAs from genes differentially expressed during the strawberry fruit ripening process by a MAST-PCR-SBDS method. *Analytical Biochemistry* **248,** 288–296.

Molina-Hidalgo FJ, Franco AR, Villatoro C, Medina-Puche L, Mercado JA, Hidalgo MA, Monfort A, Caballero JL, Muñoz-Blanco J, Blanco-Portales R. 2013. The strawberry (*Fragaria* × *ananassa*) fruit-specific *rhamnogalacturonate lyase 1* (*FaRGLyase1*) gene encodes an enzyme involved in the degradation of cell walls middle lamellae. *Journal of Experimental Botany* **64,** 1471–1483.

Moyano E, Portero-Robles I, Medina-Escobar N, Valpuesta V, Muñoz-Blanco J, Caballero JL. 1998. A fruit-specific putative dihydroflavonol 4-reductase gene is differentially expressed in strawberry during the ripening process. *Plant Physiology* **117,** 711–716.

Pedersen SB. 2001. Multiplex relative gene expression analysis by real-time RT-PCR using the iCycler iQ™ detection system. *BioRadiations (BioRad)* **107,** 10–11.

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

Perkins-Veazie P. 1995. Growth and ripening of strawberry fruit. *Horticultural Review* **17,** 267–297.

Raab T, Lopez-Ráez JA, Klein D, Caballero JL, Moyano E, Schwab W, Muñoz-Blanco J. 2006. FaQR, required for the biosynthesis of the strawberry flavor compound 4-hydroxy-2,5-dimethyl-3(2H)-furanone, encodes an enone oxidoreductase. *The Plant Cell* **18,** 1023–1037.

Ravaglia D, Espley RV, Henry-Kirk RA, Andreotti C, Ziosi V, Hellens RP, Costa G, Allan AC. 2013. Transcriptional regulation of flavonoid biosynthesis in nectarine (*Prunus persica*) by a set of R2R3 MYB transcription factors. *BMC Plant Biology* **13,** 68.

Regan BC, Julliot C, Simmen B, Vienot F, Charles-Dominique P, Mollon JD. 2001. Fruits, foliage and the evolution of primate colour vision. *Philosophical Transactions of the Royal Society B* **356,** 229–283.

Rock CD, Zeevaart JAD. 1990. Abscisic (ABA)-aldehyde is a precursor to and 1′,4′-*trans*-ABA-diol a catabolite of, ABA in apple. *Plant Physiology* **93,** 915–923.

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

Schaart JG, Dubos C, Romero-de la Fuente I, van Houwelingen AMML, de Vos RCH, Jonker HH, Xu W, Routaboul J-M, Lepiniec L, Bovy AG. 2013. Identification and characterization of MYB-bHLH-WD40 regulatory complexes controlling proanthocyanidin biosynthesis in strawberry (*Fragaria* × *ananassa*) fruits. *New Phytologist* **197,** 454–467.

Schaefer HM, Schaefer V, Levey DJ. 2004. How plant-animal interactions signal new insights in communication. *Trends in Ecology and Evolution* **19,** 577–584.

Shulaev V, Sargent DJ, Crowhurst RN *et al.* 2011. The genome of woodland strawberry (*Fragaria vesca*). *Nature Genetics* **43,** 109–118.

Spitzer-Rimon B, Marhevka E, Barkai O, Marton I, Edelbaum O, Masci T, Prathapani N-K, Shklarman E, Ovadis M, Vainstein A. 2010. *EOBII*, a gene encoding a flower-specific regulator of phenylpropanoid volatiles' biosynthesis in *Petunia*. *The Plant Cell* **22,** 1961–1976.

Spolaore S, Trainotti L, Casadoro G. 2001. A simple protocol for transient gene expression in ripe fleshy fruit mediated by *Agrobacterium*. *Journal of Experimental Botany* **52,** 845–850.

Takos AM, Jaffe FW, Jacob SR, Bogs J, Robinson SP, Walker AR. 2006. Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. *Plant Physiology* **142,** 1216–1232.

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

Walker AR, Lee E, Bogs J, McDavid DAJ, Thomas MR, Robinson SP. 2007. White grapes arose through the mutation of two similar and adjacent regulatory genes. *The Plant Journal* **49,** 772–785.

Wang Z, Meng D, Wang A, Li T, Jiang S, Cong P, Li T. 2013. The methylation of the PcMYB10 promoter is associated with green-skinned sport in Max Red Bartlett pear. *Plant Physiology* **162,** 885–896.

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

Woodward JR. 1972. Physical and chemical changes in developing strawberry fruits. *Journal of the Science of Food and Agriculture* **23,** 465–473.

Yamaguchi-Shinozaki K, Shinozaki K. 2005. Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends in Plant Science* **10,** 88–94.

Yang K, Jeong N, Moon J-K, Lee Y-H, Lee S-H, Kim HM, Hwang CH, Back K, Palmer RG, Jeong S-C. 2010. Genetic analysis of genes controlling natural variation of seed coat and flower colors in soybean. *Journal of Heredity* **101,** 757–768.

Zhang M, Yuan B, Leng P. 2009. The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *Journal of Experimental Botany* **60,** 1579–1588.