

# An R2R3-MYB Transcription Factor Regulates Eugenol Production in Ripe Strawberry Fruit Receptacles<sup>1</sup>

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Eugenol is a volatile phenylpropanoid that contributes to flower and ripe fruit scent. In ripe strawberry (*Fragaria × ananassa*) fruit receptacles, eugenol is biosynthesized by eugenol synthase (*FaEGS2*). However, the transcriptional regulation of this process is still unknown. We have identified and functionally characterized an R2R3 MYB transcription factor (*EMISSION OF BENZENOID II* [*FaEOBII*]) that seems to be the orthologous gene of *PhEOBII* from *Petunia hybrida*, which contributes to the regulation of eugenol biosynthesis in petals. The expression of *FaEOBII* was ripening related and fruit receptacle specific, although high expression values were also found in petals. This expression pattern of *FaEOBII* correlated with eugenol content in both fruit receptacle and petals. The expression of *FaEOBII* was repressed by auxins and activated by abscisic acid, in parallel to the ripening process. In ripe strawberry receptacles, where the expression of *FaEOBII* was silenced, the expression of *CINNAMYL ALCOHOL DEHYDROGENASE1* and *FaEGS2*, two structural genes involved in eugenol production, was down-regulated. A subsequent decrease in eugenol content in ripe receptacles was also observed, confirming the involvement of *FaEOBII* in eugenol metabolism. Additionally, the expression of *FaEOBII* was under the control of *FaMYB10*, another R2R3 MYB transcription factor that regulates the early and late biosynthetic genes from the flavonoid/phenylpropanoid pathway. In parallel, the amount of eugenol in *FaMYB10*-silenced receptacles was also diminished. Taken together, these data indicate that *FaEOBII* plays a regulating role in the volatile phenylpropanoid pathway gene expression that gives rise to eugenol production in ripe strawberry receptacles.

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L.M.-P. conducted most of the experiments, drafted the article, made the figures, tables, and supplementary material, and contributed to the discussion regarding the results obtained; F.J.M.-H. collaborated on the experiments of subcellular location and protein purification and participated in the validation of the microarray; M.B. collaborated on the transient transactivation assays of promoters and quantification of eugenol; R.C.S. supervised and advised on the performance of the experiments conducted by M.B. and L.M.-P.; M.B. and R.C.S. reviewed the article; I.L.-V., R.S., and J.-M.F.-Z. conducted the experiments on the determination of *FaEOBII* DNA-binding specificity and wrote part of the article; J.L.C. contributed to the analysis and interpretation of microarray data; R.B.-P. coconducted the work and carried out the isolation and cloning of the CAD promoter; J.M.-B. conducted the work, conceived the study, directed its design, contributed to the discussion of the results, and drafted the article.

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The octoploid cultivated strawberry (*Fragaria × ananassa*) is one of the most economically important, nonclimacteric, soft fruits, in which volatile compounds influence fruit flavor and aroma. Both characteristics contribute to the fruit organoleptic traits and are crucial factors to determine fruit quality.

At present, extensive surveys on the components that contribute to strawberry flavor have been performed. In these studies, more than 360 volatiles have been identified (Latrasse, 1991; Nijssen, 1996; Zabetakis and Holden, 1997), but only 15 to 20 of them in wild varieties of strawberry are believed to be essential for sensory quality, together with nonvolatile sugars and organic acids (Schieberle and Hofmann, 1997). In contrast, in cultivated varieties of strawberry, only about six odor-active compounds have been identified as contributors to fruit flavor (Raab et al., 2006; Ulrich et al., 2007). Strawberry aroma is the result of the combined perception of fruity (ethyl butanoate, ethyl hexanoate, and methyl 2-methylbutanoate), green (*Z*-3-hexenal), sweaty (butanoic acid and 2-methylbutanoic acid), peach-like ( $\gamma$ -decalactone), and caramel-like [4-hydroxy-2,5-dimethyl-3(2H)-furanone and 2,5-dimethyl-4-methoxy-3(2H)-furanone] flavor notes (Pyyalo et al., 1979; Larsen and Poll, 1992). Several wild strawberry studies have identified some of the key

components of strawberry flavor, including acetates of hydroxycinnamoyl alcohols and phenylpropenes, such as eugenol and isoeugenol, which are phenylpropanoid derivatives (Pyysalo et al., 1979; Ulrich et al., 1995, 2007). These volatile compounds, together with volatile benzenoids, such as phenylacetaldehyde, phenylethylalcohol, benzaldehyde, benzyl alcohol, benzyl benzoate, methyl benzoate, benzylacetate, methyl salicylate, and vanillin, are also floral scent components (Schuurink et al., 2006).

To date, few transcription factors (TFs) that regulate the expression of the volatile benzenoid/phenylpropanoid pathway structural genes have been identified. In petunia (*Petunia hybrida*), the function of EMISSION OF BENZENOID II (PhEOBII) and ODORANT1 (ODO1), two R2R3-MYB TFs, have been described as regulators of the volatile and nonvolatile phenylpropanoid biosynthesis pathway (Verdonk et al., 2005; Spitzer-Rimon et al., 2010). PhEOBII binds and activates the promoter of *ODO1* through the union to a MYB-binding site (MBS), indicating a hierarchical link between both TFs (Van Moerkercke et al., 2011). Previous work carried out on protoplast extracted from *Arabidopsis* (*Arabidopsis thaliana*) leaves showed binding of PhEOBII to the petunia *ISOEUGENOL SYNTHASE* (*IGS*) and to the tobacco (*Nicotiana tabacum*) Phe ammonia lyase B promoters (Spitzer-Rimon et al., 2010). Reduction of *EOBII* transcript levels in petunia petals by virus-induced gene silencing leads to a down-regulation of the expression of genes involved in the shikimate and phenylpropanoid pathways, such as *CHORISMATE SYNTHASE* (*CS*), *CHORISMATE MUTASE* (*CM*), *PHENYLALANINE AMMONIA LYASE* (*PAL2*), *CONIFERYL ALCOHOL ACETYLTRANSFERASE* (*CFAT*), *IGS*, *BENZOYL-COENZYME A:BENZYL ALCOHOL/PHENYLETHANOL BENZOYLTRANSFERASE* (*BPBT*), and *ODO1*, whereas no changes were detected in the expression levels of *CHALCONE ISOMERASE* (*CHI*) or *FLAVANONE 3-HYDROXYLASE* (*FHT*). However, overexpression of *EOBII* did not produce an increase in the expression levels of *ODO1*, *BPBT*, *CS*, *CM*, and *FHT*. This suggests that these genes could be regulated by the synergistic action of *EOBII* and other TFs. On the other hand, *PAL*, *CFAT*, and *IGS* transcripts were up-regulated, indicating a direct positive regulatory role of the expression of these genes by *EOBII* (Spitzer-Rimon et al., 2010). Besides, *ODO1* was demonstrated to activate the promoter of *5-ENOL-PYRUVYL-SHIKIMATE-3-PHOSPHATE SYNTHASE* (*EPSPS*), hence regulating the floral shikimate pathway that produces benzenoid/phenylpropanoid volatiles (Verdonk et al., 2005). Additionally, *ODO1* silencing resulted in a severe decrease of volatile production in petunia flowers, such as phenylacetaldehyde, phenylethylalcohol, methyl benzoate, benzyl acetate, benzyl benzoate, vanillin, and isoeugenol, but it did not affect the production of Phe-derived flavonols and anthocyanins (Verdonk et al., 2005). Ectopic expression of *PhODO1* in tomato (*Solanum lycopersicum*) increased the expression of only a subset of phenylpropanoid-associated genes. Changes in the expression of genes related to flavonoid synthesis were not detected in transgenic fruits. These data

were consistent with the metabolomic changes observed (Dal Cin et al., 2011).

Recently, a third TF (*EOBI*) has been identified in petunia as a regulator of floral scent production through direct modulation of the expression of *ODO1* and structural scent-related genes. *EOBI* is an R2R3-MYB TF that is required for the proper expression of structural and regulatory genes related to floral phenylpropanoid scent production (Spitzer-Rimon et al., 2012). *EOBI* silencing down-regulated several genes from the shikimate and phenylpropanoid pathways (i.e. *EPSPS*, *CS*, *CM*, *PAL*, *IGS*, *EGS*, and *ODO1*, among others), which clearly led to reduced levels of floral volatiles, such as benzylbenzoate, phenylethylbenzoate, benzyl alcohol, methylbenzoate, methylsalicylate, isoeugenol, and eugenol (Spitzer-Rimon et al., 2012). However, an *EOBI* orthologous gene has not been detected in strawberry yet.

Eugenol and isoeugenol are volatile compounds produced by plants as floral attractants of pollinators as well as defense compounds (Koeduka et al., 2006; Pasay et al., 2010). Fruits can also synthesize volatile phenylpropenes that contribute to their aroma (Jordán et al., 2001; Aubert and Pitrat, 2006; Ortiz-Serrano and Gil, 2010). Eugenol production by ripe strawberry fruits has been reported previously (Pyysalo et al., 1979; Zorrilla-Fontanesi et al., 2012). Interestingly, the amount of this volatile is lower in cultivated fruits (*F. ananassa*) than in wild diploid *Fragaria vesca* (Pyysalo et al., 1979). By contrast, isoeugenol biosynthesis in strawberry fruits is extremely low in comparison with eugenol levels (Hoffmann et al., 2011).

The last step of the phenylpropene eugenol biosynthesis is catalyzed by *EUGENOL SYNTHASE* (*EGS*), an NADPH-dependent reductase belonging to the PIP (for Pinoresinol-lariciresinol reductase, Isoflavone reductase, Phenylcoumaran benzylic ether reductase) family (Min et al., 2003). To date, only a small number of plant *EGS*s, such as *ObEGS1* (from *Ocimum basilicum*; Koeduka et al., 2006), *CbEGS1* and *CbEGS2* (from *Clarkia breweri*), and *PhEGS1* (from petunia; Koeduka et al., 2008), have been functionally characterized. These enzymes can use coniferyl acetate as a substrate for eugenol in vitro production. Overexpression of a coniferyl alcohol acetyltransferase (*PhCFAT*) and *PhEGS* from petunia in aspen (*Populus* spp.) increased eugenol production in leaves (Koeduka et al., 2013).

More recently, two different genes (*FaEGS1* and *FaEGS2*) coding two strawberry eugenol synthases have been functionally characterized (Aragüez et al., 2013). *FaEGS1* was predominantly expressed in green achenes, whereas the expression of *FaEGS2* was specific to ripe fruit receptacles (Aragüez et al., 2013). The expression pattern of both genes correlated with eugenol content of both achenes and fruit receptacles. *FaEGS1* as well as *FaEGS2* enzymes can use in vitro coniferyl acetate as a substrate for eugenol production (Aragüez et al., 2013). Although eugenol production in strawberry fruit has been clarified, the regulation of the volatile benzenoid/phenylpropanoid structural pathway that renders eugenol in strawberry fruit receptacles has not been elucidated.

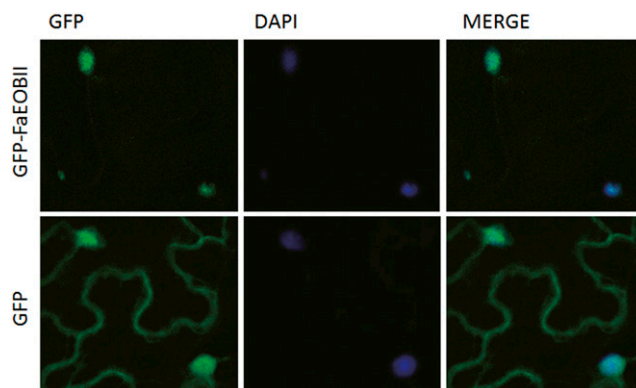
Previously performed transcriptomic studies in our research group allowed us to identify a wide group of genes whose expression increased throughout strawberry fruit ripening. One of these genes, *FaEOBII* (*gene28435*, 46.063 up), showed a significant sequence homology with the R2R3-MYB, such as the TF *PhEOBII*. Nevertheless, we have not observed the up-regulation of any orthologous *ODO1* or *EOBI* genes when comparing the transcriptomes of mature red-ripe and immature green receptacles. Although a putative orthologous gene of *EOBI* from petunia has not been detected in the strawberry genome, putative orthologous genes of *ODO1* from petunia have been identified in the strawberry genome (*gene32268*, *gene21264*, *gene12564*, and *gene30520*). However, none of them present an expression pattern associated with the ripening process of the strawberry receptacles (*gene21264*, 1.559 down; *gene12564*, 1.468 down; and *gene30520*, 1.024 up), but the expression of *gene32268* (*FaODO1*), by both microarray studies (13.875 down) and quantitative real-time (qRT)-PCR (23.912 down), was down-regulated during the ripening process.

Here, we present the functional characterization of the R2R3-MYB TF *FaEOBII*, whose expression is fruit receptacle specific and that is related to eugenol production. This regulatory role is executed through the activation of *CINNAMYL ALCOHOL DEHYDROGENASE* (*FaCAD1*) and *FaEGS2* gene expression.

## RESULTS

### Sequence Analysis of the *FaEOBII* Gene and Protein

The full-length complementary DNA (cDNA) sequence of the *FaEOBII* gene contained an open reading frame of 624 bp that encodes a polypeptide of 207 amino acid residues and has a predicted molecular mass of 23.26 kD. WoLF PSORT predicted the nuclear location of this protein (Supplemental Fig. S1). To confirm this hypothesis, we have determined the subcellular location of the *FaEOBII* protein in vivo. To that end, an N-terminal translational fusion protein construct between *FaEOBII* and GFP was transiently expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in *Nicotiana benthamiana* leaves. As expected, confocal imaging of the fusion protein revealed colocalization with the nucleus marker 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) in the parenchyma cells of the abaxial epidermis of tobacco leaves, thus confirming the nuclear location of the *FaEOBII* protein (Fig. 1). The analysis of the predicted *FaEOBII* protein revealed the R2 and R3 repeat signatures at its N terminus that are characteristic of the R2R3 DNA-binding MYB proteins (Kranz et al., 1998; Supplemental Fig. S1), which are important for interaction with regulatory sequences in promoters (Kranz et al., 1998; Dubos et al., 2010). We also identified a W/Y-MDDIW transactivation motif (Li et al., 2006) at its C terminus, with three particular amino acids (Trp-193, Asp-197, and Trp-199) conserved in all the sequences analyzed (Supplemental Fig. S2). Based on

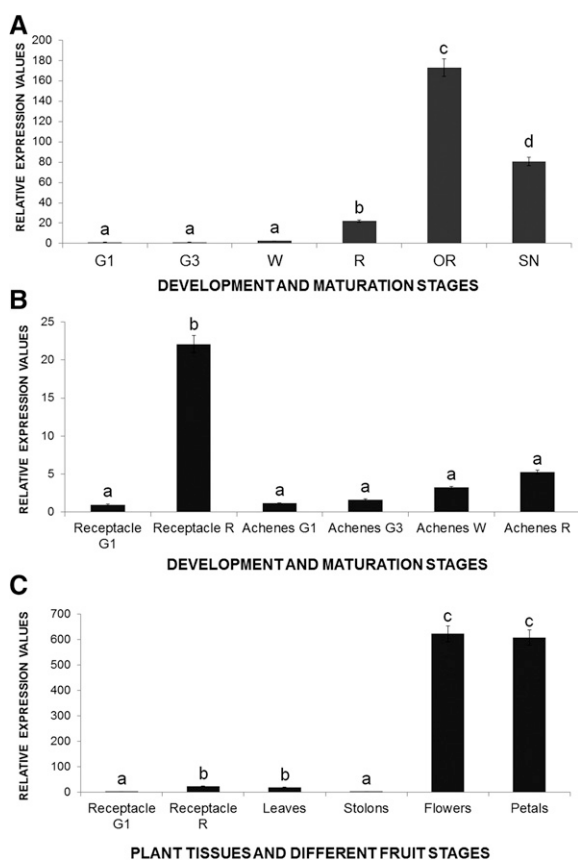


**Figure 1.** Nuclear location of the *FaEOBII* protein in plant cells. Fluorescence signal was detected using a confocal microscope from GFP-*FaEOBII* (top row) and GFP (bottom row) expression under the control of the 35S promoter in *N. benthamiana* leaf epidermis cells. The MERGE column shows merged views of the GFP and DAPI images.

these features outside the DNA-binding R2R3 domain, *FaEOBII* can be classified into subgroup 19 of the R2R3-MYB TFs (Dubos et al., 2010; Supplemental Fig. S3; Supplemental Tables S1 and S2). Phylogenetically, *FaEOBII* shares high amino acid homology with members of this subgroup of MYB TFs from other plants, such as PsMYB26 (*Pisum sativum*), NlxNsMYB305 (*Nicotiana langsdorffii* × *Nicotiana sanderae*), AmMYB305 (*Antirrhinum major*), AmMYB340 (*A. major*), AtMYB24 (*Arabidopsis*), AtMYB21 (*Arabidopsis*), and *PhEOBII* (petunia). Functional characterization of all of these TFs has demonstrated that they are involved in the regulation of the volatile benzenoid phenylpropanoid metabolic pathway (Sablowski et al., 1994; Moyano et al., 1996; Uimari and Strommer, 1997; Shin et al., 2002; Li et al., 2006; Liu et al., 2009; Spitzer-Rimon et al., 2010). All of these bioinformatic analyses suggested that *FaEOBII* could be an R2R3 MYB TF potentially involved in the regulation of those metabolic pathways related to volatile phenylpropanoid production in ripe receptacles.

### The Expression of *FaEOBII* Is Highly Expressed in Ripe Fruit Receptacles and Petals and Correlates with Eugenol Production in Both Tissues

*FaEOBII* was expressed at very low levels during fruit receptacle elongation at early developmental stages, experiencing an increase in receptacle ripening stages, with a maximum peak taking place in the overripe stage. This maximum expression was followed by a decrease in expression in the senescent stage (Fig. 2A). On the other hand, when compared with those expression values obtained in fruit receptacle at the red stage, *FaEOBII* was weakly expressed in fruit achenes in all developmental and ripening stages studied (Fig. 2B). This low expression was also observed in other vegetative tissues analyzed, such as leaves and stolons. However, in flowers and specifically in petals, the amount of *FaEOBII* transcript was higher than in ripe fruits (Fig. 2C).

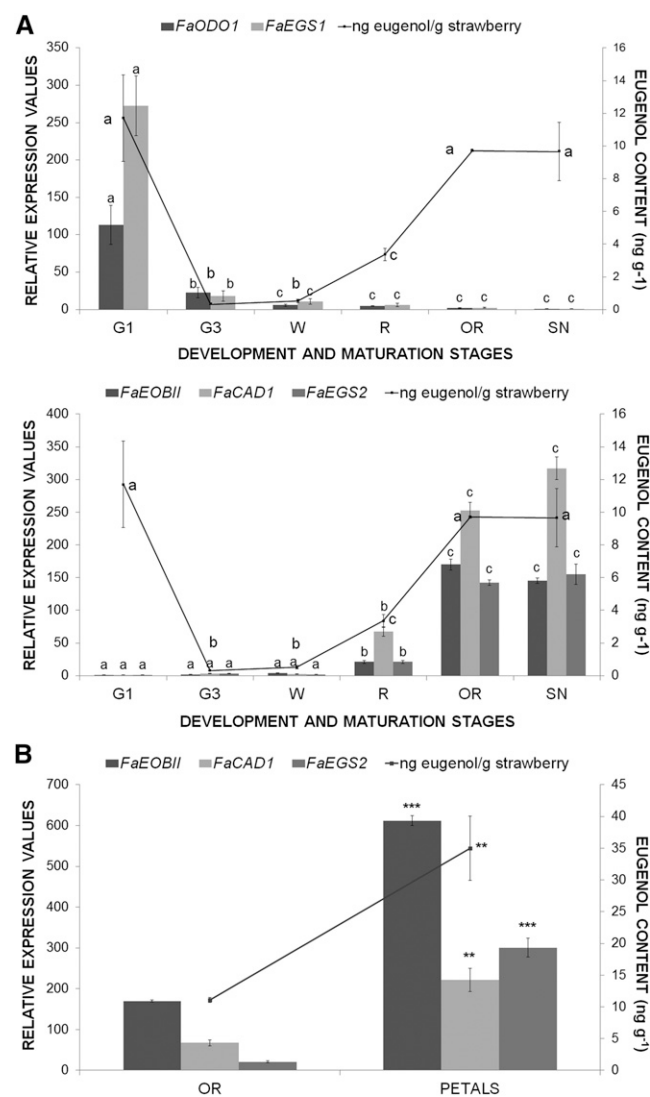


**Figure 2.** Analysis by qRT-PCR of *FaEOBII* expression in developing fruit receptacles (A), achenes (B), and plant tissues (C) of *F. ananassa* 'Camarosa.' G1, Small-sized green fruit; G3, full-sized green fruit; W, white stage; R, ripe stage; OR, overripe stage; SN, senescent stage. Quantification is based on cycle threshold (C<sub>t</sub>) values. Relative expression values were calculated relative to receptacles in the G1 stage in all cases, which was assigned an arbitrary value equal to unity. Values are means  $\pm$  SD of five independent experiments. Statistical significance was determined by one-way ANOVA. Letters indicate significant differences ( $P < 0.05$ , Scheffe's post hoc test).

These results showed that *FaEOBII* expression was not only up-regulated in ripe fruit receptacles but also in petals.

It was shown that throughout the strawberry fruit-ripening process, eugenol accumulates in ripe receptacles (Pyysalo et al., 1979; Zorrilla-Fontanesi et al., 2012; Aragüez et al., 2013). The orthologous gene of *FaEOBII* in petunia (*PhEOBII*) has been shown to be involved in eugenol production (Spitzer-Rimon et al., 2010). Thus, we analyzed eugenol production in strawberry fruit receptacles during ripening (Fig. 3A) as well as in those plant tissues where a higher *FaEOBII* gene expression was observed (Fig. 3B). A clear connection between high *FaEOBII* gene expression during the ripening stages and high eugenol content was found. In fact, the highest levels of *FaEOBII* gene expression and of eugenol content were observed in red overripe receptacles and in petals (Fig. 3B). Recently, eugenol production in unripe strawberry fruits has been reported to be mainly restricted to

achenes at the green stage (Aragüez et al., 2013) as well as coincident with the expression pattern of *FaEGS1* in achenes (Aragüez et al., 2013). Our results indicate that there is also eugenol production in young unripe green receptacles that is coincident with the *FaEGS1* gene expression but not with the expression of *FaEOBII* (Fig. 3A), discounting a regulatory role of *FaEOBII* in *FaEGS1* gene expression. However, in green unripe fruit receptacles, we detected the presence of an ODO1 orthologous gene



**Figure 3.** Analysis of spatiotemporal changes in gene expression and content of eugenol. A, Bars represent the developmental expression of strawberry *FaODO1*, *FaEGS1*, *FaEOBII*, *FaCAD1*, and *FaEGS2* genes in receptacles of *F. ananassa* 'Camarosa' obtained by qRT-PCR. B, Bars represent relative expression values of *FaEOBII*, *FaCAD1*, and *FaEGS2* genes in overripe receptacles versus petals. Stages are as defined in Figure 2. Lines represent gas chromatography (GC)-mass spectrometry (MS) quantification of eugenol. Values are given in nanograms of eugenol per gram of strawberry. One-way ANOVA was performed on log-transformed data, and letters indicate significant differences ( $P < 0.05$ , LSD post hoc test).

(Fig. 3A) that could regulate the expression of *FaEGS1* in a similar way to what is described for *PhODO1* (Verdonk et al., 2005). *FaCAD1* encodes a cinnamyl alcohol dehydrogenase that produces cinnamyl alcohols that are, in turn, the substrate for the production of volatile phenylpropanoids such as eugenol. This enzyme works upstream of the *FaEGS2* enzyme in the metabolic pathway that renders eugenol. Thus, for this reason, *FaCAD1* and *FaEGS2* genes have been included in the expression studies. Figure 3A showed a clear correlation in the expression profile of *FaEOBII*, *FaCAD1*, and *FaEGS2* during the ripening process of strawberry fruit receptacles as well as a concomitant increase in the eugenol content during maturation stages.

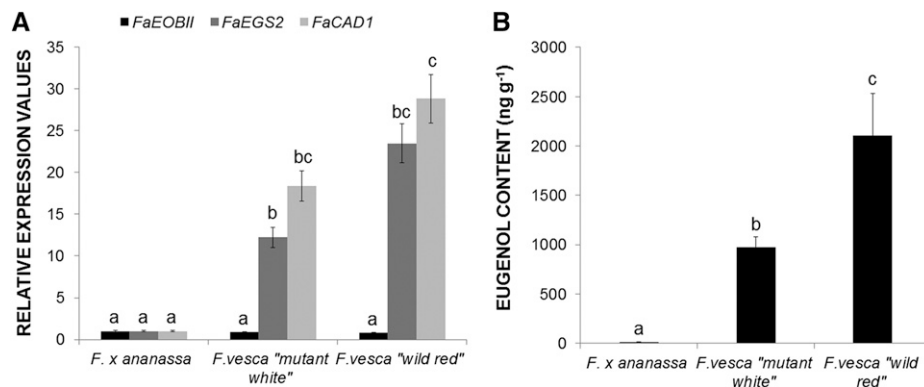
Interestingly, in strawberry fruit receptacles, the expression pattern of *FaEOBII* was quite similar to that observed for *FaEGS2*. Similarly, eugenol content, as well as *FaCAD1* and *FaEGS2* gene expression, were higher in *F. vesca* than in *F. ananassa* ripe fruits. In contrast, the expression of *FaEOBII* was unchanged between both *Fragaria* spp. (Fig. 4). Using the PLACE server (<http://www.dna.affrc.go.jp/PLACE/>), several MBSII boxes recognized by the *FaEOBII* TF were subsequently located in the *EGS2* promoter sequences (Supplemental Fig. S4). In view of this bioinformatic analysis of promoter sequences, the *FvEGS2* promoter contains more MBSII boxes than the *FaEGS2* promoter.

#### The Expression of *FaEOBII* Is Hormonally Regulated throughout Receptacle Fruit Growth and Ripening

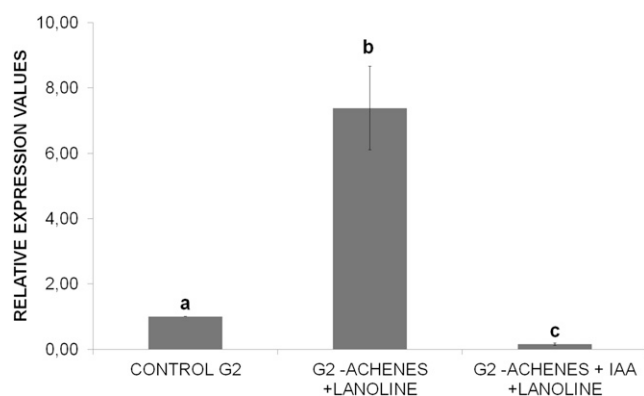
It has been proposed that the abscisic acid (ABA)-auxin ratio in strawberry fruit receptacles constitutes the signal that triggers the fruit-ripening process (Perkins-Veazie, 1995; Jiang and Joyce, 2003). According to this

proposal, the auxins that are produced by achenes and released to the fruit receptacle would repress the expression of a vast majority of the ripening-related genes (Medina-Escobar et al., 1997; Manning, 1998; Moyano et al., 1998; Blanco-Portales et al., 2002, 2004; Raab et al., 2006; Medina-Puche et al., 2014). To determine whether *FaEOBII* gene expression was under auxin control, we determined the changes in *FaEOBII* gene expression in unripe fruit receptacles after removing the achenes from the fruit surface. This treatment reduces the endogenous content of auxins in the fruit receptacle. After 5 d of treatment, the *FaEOBII* expression level was clearly increased when compared with what had been observed in untreated control fruits. This increase in the amount of *FaEOBII* transcript in deachened fruits was abolished by the external application of auxin (indole-3-acetic acid [IAA]), thus indicating that this gene expression is under the negative control of auxins (Fig. 5).

Recent reports have suggested that the phytohormone ABA may be involved in the positive regulation of anthocyanin production by promoting strawberry fruit ripening (Chai et al., 2011; Jia et al., 2011; Medina-Puche et al., 2014). To ascertain if this phytohormone regulates *FaEOBII* gene expression, we modulated the internal receptacle ABA content in three different experimental conditions: (1) by injecting into the interior of fruit receptacle nordihydroguaiaretic acid (NDGA), an inhibitor of the 9-CIS-EPOXYCAROTENOID DIOXYGENASE (NCED) activity, the key enzyme responsible for ABA biosynthesis (Fig. 6A); (2) by transiently silencing *FaNCED1* expression through in vivo agroinfiltration (Fig. 6B); and (3) by depleting water from plants, as water stress is known to increase ABA content in plants (Fig. 6C). Under all conditions, a clear variation in the expression of *FaEOBII* parallel to the endogenous receptacle ABA content was observed



**Figure 4.** Analysis of gene expression changes and eugenol content. A, Analysis by qRT-PCR of *EOBII*, *EGS2*, and *CAD1* gene expression in red ripe receptacles of the cultivated strawberry *F. ananassa* 'Camarosa' and in the wild strawberries *F. vesca* Mutant White and *F. vesca* Wild Red of cv Reina de los Valles. Quantification is based on  $C_t$  values as described in "Materials and Methods." The increase in the mRNA value was relative to the *F. ananassa*  $C_t$  value of each experiment, which was assigned an arbitrary value equal to unity. Mean values  $\pm$  sd of six independent experiments are shown. Statistical significance was determined by one-way ANOVA. Letters indicate significant differences ( $P < 0.05$ , Scheffe's post hoc test). B, GC-MS quantification of eugenol in strawberry fruit varieties. Values are given in nanograms of eugenol per gram of strawberry. One-way ANOVA was performed on log-transformed data, and letters indicate significant differences ( $P < 0.05$ , LSD post hoc test).



**Figure 5.** Analysis of the effects of removing achenes from G2 developing fruits of *F. ananassa* ‘Camarosa’ on *FaEOBII* expression by qRT-PCR. Quantification is based on  $C_t$  values as described in “Materials and Methods.” Increase in the mRNA value was relative to the receptacle G2  $C_t$  value of each experiment, which was assigned an arbitrary value equal to unity. Values are means  $\pm$  SD of five independent experiments. CONTROL G2, Middle-sized green fruit receptacle; G2-ACHENES + LANOLINE, G2 fruit receptacles without achenes for 5 d covered by a lanolin paste; G2-ACHENES + IAA + LANOLINE, G2 fruit receptacle without achenes for 5 d treated with the synthetic auxin IAA as a lanolin paste with 1 mM IAA. Statistical significance was determined by one-way ANOVA. Letters indicate significant differences ( $P < 0.05$ , Scheffé’s post hoc test).

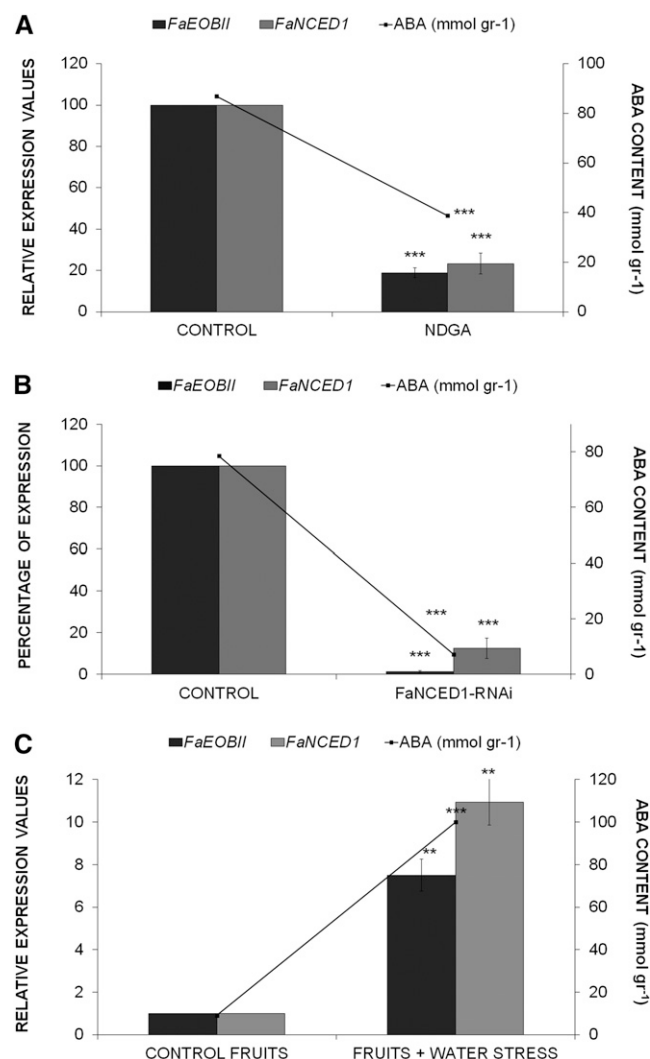
(Fig. 6; Supplemental Fig. S5). These results strongly suggest that ABA can positively regulate the expression of the *FaEOBII* gene.

Taken together, these results indicate that *FaEOBII* 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), Jia et al. (2011), and Medina-Puche et al. (2014).

### FaEOBII Can Transactivate the Promoters of Genes Involved in Volatile Phenylpropene Production in Strawberry Fruit Receptacles

The potential regulatory role played by *FaEOBII* in the expression of the structural genes belonging to the volatile phenylpropanoid biosynthesis pathway in ripe strawberry receptacles was assessed through transcriptomic studies. For this purpose, the expression of *FaEOBII* was transiently down-regulated in fruit receptacles by an RNA interference (RNAi) approach. The silencing degree was measured in fruit receptacle by qRT-PCR 14 d after agroinjection. Analyzed transgenic fruits showed an important reduction in *FaEOBII* expression ranging between 88.3% and 99.5%, whereas no changes in *FaEOBII* expression were found in control fruits (Figs. 7B and 8B). Fruit receptacles that exhibited a silencing percentage above 99% were used for transcriptomics analysis. A custom-made oligonucleotide-based microarray platform representing 34,496 putative

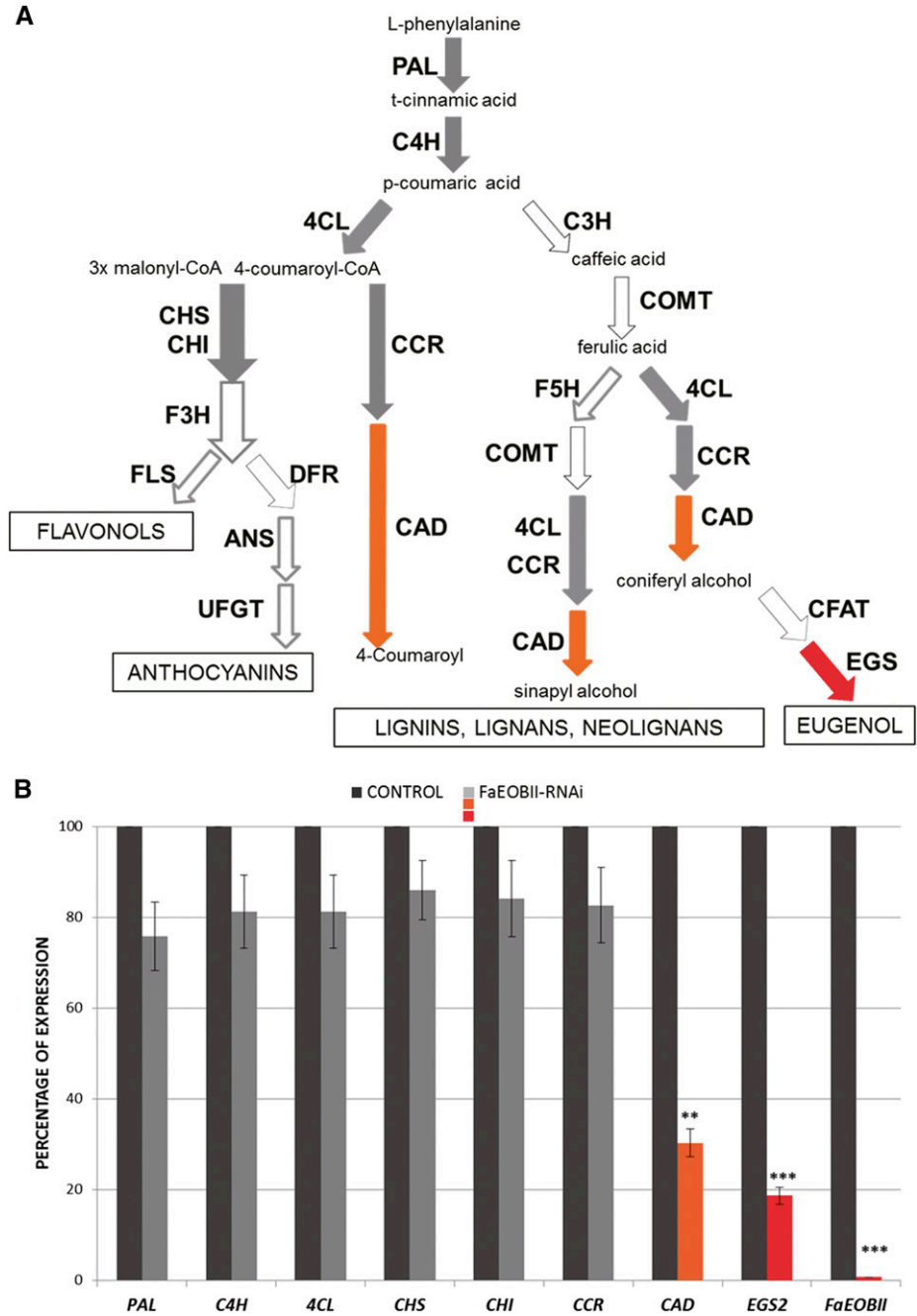
genes from the *Fragaria* spp. genome (FraGenomics35K) was used. Transcriptome analyses were carried out to compare gene expression profiles of transgenic transiently silenced *FaEOBII* fruits against control fruits that were agroinfiltrated with the empty pFRN vector. The work focused on the most clearly up- and down-regulated genes by choosing a 2-fold cutoff and a corrected  $P$  value of less than 0.01. This microarray analysis revealed that only 10 transcripts were down-regulated, whereas no transcript was up-regulated in



**Figure 6.** Analysis by qRT-PCR of *FaEOBII* and *FaNCED1* expression (bars) and quantification of ABA concentration (lines). A, CONTROL, Green-white fruits injected with water; NDGA, green-white fruits injected with NDGA (100  $\mu$ M). B, CONTROL, Control fruits agroinfiltrated with the empty pFRN vector; FaNCED1-RNAi, transgenic strawberry fruits agroinfiltrated with the pFRN-*FaNCED1* construct. C, CONTROL FRUITS, Fruits with the pedicels immersed in Murashige and Skoog medium with Suc; FRUITS + WATER STRESS, fruits with their pedicels kept in the air. Mean values  $\pm$  SD of five independent experiments are shown. Statistical significance with respect to the reference sample (CONTROL) was determined by Student’s  $t$  test: \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$ .



**Figure 7.** *FaEOBII* silencing effect on the expression of the structural genes belonging to the volatile phenylpropanoid biosynthesis pathway. A, Schematic diagram of the phenylpropanoid pathway in plants. White arrows indicate nonanalyzed genes. Gray arrows represent genes with no significant transcriptomic changes when *FaEOBII* is silenced. Orange and red arrows represent genes with significant transcriptomic changes in *FaEOBII*-silenced fruits. C4H, Cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase (also called LDOX, leucoanthocyanidin dioxygenase); UFGT, UDP-flavonoid glucosyltransferase; C3H, *p*-coumarate 3-hydroxylase; COMT, caffeic *O*-methyltransferase; F5H, ferulic acid 5-hydroxylase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase. B, Analysis by qRT-PCR of the expression of genes involved in the phenylpropanoid pathway in *FaEOBII*-silenced fruits agroinfiltrated with the pFRN-*FaEOBII* construct, compared with control fruits agroinfiltrated with the empty pFRN vector only. The silencing level is expressed as a percentage. Statistical significance with respect to the reference sample (CONTROL) was determined by Student's *t* test: \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$ .



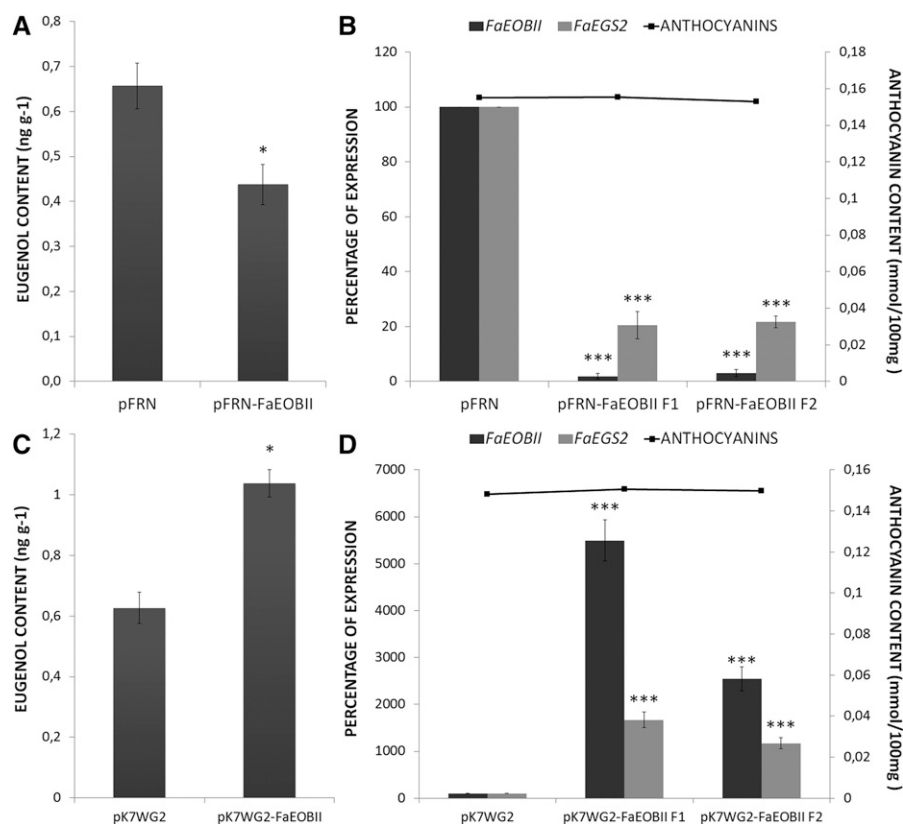
transiently *FaEOBII*-silenced fruits (Table I; Supplemental Tables S3 and S4). Two of them, *FaCAD1* (*gene20700*) and *FaEGS2* (*gene25260*), encode enzymes and were involved in eugenol biosynthesis (Fig. 7A). This down-regulation was also corroborated by qRT-PCR (Fig. 7B; Supplemental Table S4). For this reason, eugenol content in *FaEOBII*-silenced fruits was analyzed. The results showed a significant decrease of eugenol content (Fig. 8A) in *FaEOBII*-silenced fruits, whereas anthocyanin content remained unchanged (Fig. 8B).

In parallel, the expression of *FaEOBII* was transiently up-regulated in fruit receptacles by overexpression under the control of the CaMV 35S promoter. The increased

expression was measured by qRT-PCR, and the results showed a statistically significant increase in both eugenol content (Fig. 8C) and *FaEOBII* and *FaEGS2* gene expression (Fig. 8D) in the *FaEOBII* overexpression construct receptacles, whereas no changes were detected in the anthocyanin content (Fig. 8D).

#### **FaEOBII Can Activate the Promoter of *FvCAD1* That Encodes a Key Enzyme of Eugenol Metabolism**

In order to determine the *in vivo* ability to trans-activate the promoters of *FaCAD1* and *FaEGS2*, we



**Figure 8.** GC-MS quantification of eugenol, qRT-PCR analysis, and anthocyanin measurements in *FaEOBII* transitory transgenic fruit receptacles. A and C, GC-MS quantification of eugenol in strawberry fruit receptacles with *FaEOBII* expression silenced (pFRN-*FaEOBII*) with respect to control fruits (pFRN; A) and *FaEOBII* expression increased (pK7WG2-*FaEOBII*) with respect to control fruits (pK7WG2; C). Values are given in nanograms per gram. Statistical significance with respect to the reference sample (pFRN or pK7WG2) was determined by Student's *t* test: \*,  $P < 0.05$ . B and D, Bars represent the analysis by qRT-PCR of *FaEOBII* and *FaEGS2* expression in transgenic strawberry fruits agroinfiltrated with the pFRN-*FaEOBII* construct and in control fruits agroinfiltrated with the empty pFRN vector (B) and with the pK7WG2-*FaEOBII* construct and in control fruits agroinfiltrated with the empty pK7WG2 vector (D). The silencing and overexpression levels are expressed as percentages. Quantification is based on  $C_t$  values as described in "Materials and Methods." The values refer to the mRNA  $C_t$  value of control samples, which were assigned an arbitrary value equal to unity. Mean values  $\pm$  SD of three independent experiments are shown. Lines represent anthocyanin content quantified on fruits analyzed. Statistical significance with respect to the reference sample (pFRN or pK7WG2) was determined by Student's *t* test: \*\*\*,  $P < 0.001$ .

conducted transitory assays on *N. benthamiana* leaves. After several efforts to clone the *FaEGS2* promoter, we did not succeed. For this reason, in its place we used the *FvCAD1* gene promoter (Fig. 9A) for this assay. For this purpose, we performed transient transactivation assays of the *FvCAD1* promoter from *F. vesca* with the *FaEOBII* TF in *N. benthamiana* leaves. The *pCAD1*:GUS reporter construct was expressed together with the effector construct 35S:*FaEOBII*. As a control, the same reporter construct was separately coinfiltrated with the R2R3-MYB TFs EOBII (*PhEOBII*) and ANTHOCYANIN2 (AN2) effector constructs from petunia (Fig. 9B). An 8-fold increase in reporter gene activation was observed when coinfiltration with *FaEOBII* was compared with control infiltration (AN2). Lower induction values (up to 4.6-fold) were detected with *PhEOBII* as effector (Fig. 9C). These results indicate that *FaEOBII* activates the *FvCAD1* promoter in *N. benthamiana* leaves.

As a positive control in this experiment, the trans-activation of the *PhODO1* promoter (Van Moerkercke et al., 2011) by *FaEOBII* was carried out. The results obtained show the accurate completion of the experiment (Fig. 9, D–F).

#### **FaEOBII Binds to MYB-Binding Sequences in Vitro**

To analyze whether *FaEOBII* could bind to MYB-binding DNA sequences, we determined the DNA-binding specificity of *FaEOBII* using the PBM11 microarray, which contains all possible 11-mer sequences (Godoy et al., 2011). After array incubation and data processing (Berger and Bulyk, 2009; Godoy et al., 2011), we obtained the list of the top-scoring 8-mers, shorted in relation to their enrichment scores. The GTTAGGT sequence was obtained with the highest enrichment score, thus indicating that this



**Table 1.** Microarray data from a transcriptomic comparison between transgenic transiently silenced *FaEOBII* fruits and control fruits agroinfiltrated with the empty *pFRN* vector

Gene identifiers and corresponding annotations are as reported in the *F. vesca* genome database (<http://www.strawberrygenome.org/>; Shulaev et al., 2011).

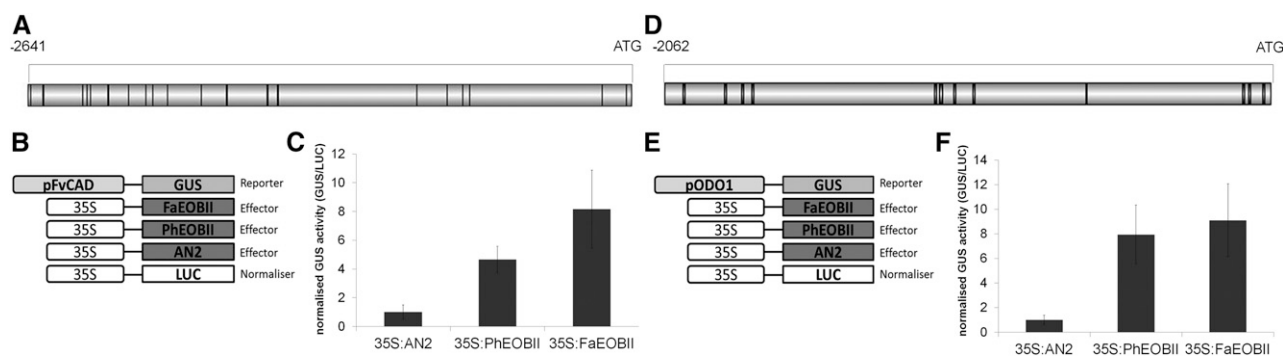
Gene Identifier	RNAi- <i>FaEOBII</i>	P	Annotation	Species	E Value	Accession No.
<i>gene28435</i>	6.549 down	0.00035	MYB TF emission of benzenoids II ( <i>FaEOBII</i> )	<i>F. ananassa</i>	0.0	KM099230
<i>gene25260</i>	4.019 down	0.00437	Eugenol synthase2 ( <i>FaEGS2</i> )	<i>F. ananassa</i>	4E-148	KF562266.1
<i>gene20700</i>	3.047 down	0.00501	Cinnamyl alcohol dehydrogenase ( <i>FaCAD1</i> )	<i>F. ananassa</i>	0.0	U63534.1
<i>gene20643</i>	2.991 down	0.00343	$\beta$ -Glucosidase3	<i>F. ananassa</i>	0.0	JX244263.1
<i>gene32526</i>	2.465 down	0.00262	DNA-binding with one finger zinc finger protein	<i>Medicago truncatula</i>	6E-49	XM_003602109.1
<i>gene25718</i>	2.208 down	0.00167	Hexokinase	<i>Eriobotrya japonica</i>	3E-179	JF414121.2
<i>gene21312</i>	2.207 down	0.002	Gland development-related protein90-like	<i>Gossypium hirsutum</i>	2E-21	EU373080.1
<i>gene12176</i>	2.202 down	0.00174	Vernalization insensitive	<i>Theobroma cacao</i>	0.0	XM_007018178.1
<i>gene18910</i>	2.150 down	0.0011	$\beta$ -Hydroxyisobutyryl-CoA hydrolase1	Arabidopsis	5E-104	NM_125991.3
<i>gene06729</i>	2.083 down	0.00487	Cytochrome P450	<i>Populus trichocarpa</i>	0.0	XM_002307093.1
<i>gene30534</i>	2.076 down	0.00498	Nonspecific lipid-transfer protein	<i>M. truncatula</i>	2E-22	XM_003620358.1

motif is recognized with the highest affinity by *FaEOBII* (Fig. 10A). This motif can be interpreted as an MBSIIg box (GKTWGGTR; R, A, or G; K, G, or T; W, A, or T; Prouse and Campbell, 2012), similar to those obtained for other plant R2R3 MYBs (Franco-Zorrilla et al., 2014). Given that some R2R3 MYBs also recognize MBSII elements, we evaluated binding to DNA motifs in which the G nucleotide in the sixth position in the GTTAGGT sequence was replaced by a T residue. In this case, the highest affinity was observed for MBSIIg motifs, in particular RTTAGGT sequences (Fig. 10B). By contrast, binding to their corresponding MBSII elements was very low, thus indicating that *FaEOBII* does not efficiently recognize these elements (Fig. 10B).

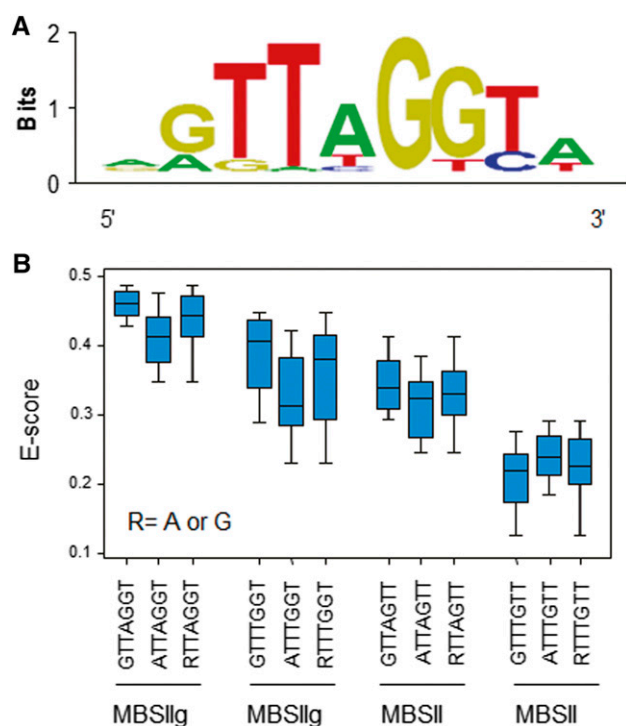
### *FaEOBII* Expression Is under the Regulation of the *FaMYB10* TF

In petunia, a flower-specific gene regulatory network has been described that constitutes the phenylpropanoid

volatile biosynthesis pathway that is controlled by three R2R3 MYB TFs, ODO1, EOBII, and EOBI (Verdonk et al., 2005; Spitzer-Rimon et al., 2010, 2012; Van Moerkercke et al., 2011). Recently, in strawberry, it was demonstrated that the transitory silencing of *FaMYB10* gene expression led to a down-regulation of structural genes involved in the flavonoid/phenylpropanoid metabolic pathway (Medina-Puche et al., 2014) as well as *FaEOBII* expression. We have corroborated these microarray results through qRT-PCR experiments. Thus, we have observed that silencing of *FaMYB10* expression resulted in a concomitant down-regulation of *FaEOBII* expression as well as a reduction of both anthocyanin and eugenol contents in the strawberry fruit receptacles. However, after silencing of *FaEOBII* expression in fruit receptacles, neither the amount of *FaMYB10* transcripts nor the anthocyanins was reduced, although the concentration of eugenol decreased significantly (Fig. 11). These results clearly indicate that *FaMYB10* regulates *FaEOBII* expression but not the contrary, and they support even more the involvement of



**Figure 9.** Transactivation of the *CAD1* promoter of *F. vesca* and the *ODO1* promoter of petunia 'Mitchell' by EOBI in *N. benthamiana* leaves. A and D, Schematic diagram of *pFvCAD1* and *pODO1* promoters using the PLACE online database ([www.dna.affrc.go.jp](http://www.dna.affrc.go.jp)) and DOG 2.0.1 for Windows (Illustrator of Protein Domain Structures). The bars at the top indicate the lengths of the promoter fragments relative to the ATG codon. The locations of the MBSII boxes are shown using black lines. B and E, Constructs used for the transactivation assays. C and F, Normalized GUS activity after coinfiltration with *A. tumefaciens* harboring any of the effector constructs with the *pFvCAD1*:GUS or the *pODO1*:GUS reporter construct. Coinfiltration with the CaMV 35S:AN2 effector was used to show EOBI specificity. Coinfiltration with 35S:LUC enabled normalization (averages  $\pm$  se).



**Figure 10.** Identification of FaEOBII-binding motifs in vitro. A, Position weight matrix representation of the top-scoring 9-mer obtained in the seed-and-wobble algorithm. B, Box plot of enrichment scores (E-scores) containing all possible 7 mers indicated representing the MBSIIg and MBSII elements. Blue boxes represent quartiles 25% to 75%, and the black line represents the median of the distribution (quartile 50%). Bars indicate quartiles 1% to 25% (above) and 75% to 100% (below).

FaEOBII in the regulation of eugenol biosynthesis in red-ripe receptacles.

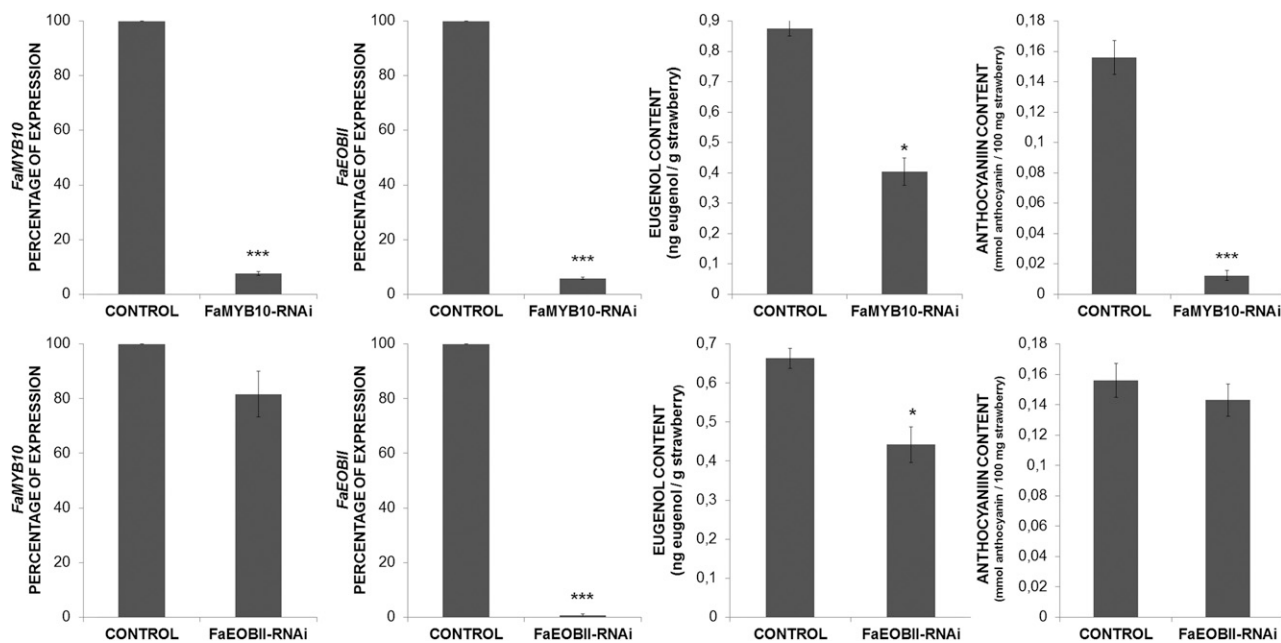
## DISCUSSION

The transcriptional regulatory network of the strawberry fruit-ripening process is poorly known. So far, the functional roles of only a low number of TFs that regulate the expression of the ripening-related genes and related metabolic pathways that determine the organoleptic characteristics of the strawberry ripe fruit have been clarified. Thus, by using a heterologous system, it was proposed that the R2R3 MYB TF FaMYB1 plays a repressor role in the expression of phenylpropanoid and anthocyanin structural genes in ripe fruit receptacles (Aharoni et al., 2001). However, transient silencing of *FaMYB1* gene expression in strawberry receptacles did not change the anthocyanin content in transgenic ripe receptacles (Medina-Puche et al., 2014). Similarly, it was demonstrated that the fruit-specific and ripening-related *FaMYB10* gene, which encodes an R2R3 MYB TF, regulates the expression of early and late structural genes of the flavonoid/phenylpropanoid metabolic pathway (Medina-Puche et al., 2014). Silencing the expression of this TF, in fruit receptacles, reduced the expression of genes involved

in anthocyanin and phenylpropanoid volatile production as well as the expression of a putative MYB TF (*FaEOBII*; Medina-Puche et al., 2014). This suggests a direct connection between *FaMYB10*, *FaEOBII*, and the flavonoid/phenylpropanoid metabolism in ripe fruits. In this article, we present the functional characterization of the role played by *FaEOBII* throughout the strawberry fruit-ripening process.

### FaEOBII Regulates the Expression of Two Structural Genes That Play a Key Role in Eugenol Biosynthesis in Fruit Receptacles

Transcriptomic and qRT-PCR assays in fruit receptacles where the expression of *FaEOBII* was silenced by RNAi showed a decrease in the expression of a small subset of ripening-related genes (Fig. 7B; Table I; Supplemental Tables S3 and S4). Among them, we found two genes, *FaCAD1* (*gene20700*, coding a cinnamyl alcohol dehydrogenase; Blanco-Portales et al., 2002) and *FaEGS2* (*gene25260*, coding an eugenol synthase; Aragüez et al., 2013), that encode for two key enzymes of the metabolic pathway that produces eugenol. FaCAD1 is an enzyme that catalyzes the production of monolignols that are precursors of phenylpropenes, such as eugenol. Afterward, these monolignol alcohols are acetylated in the hydroxyl group by a coniferyl alcohol acyl transferase. FaEGS2 catalyzes the reductive cleavage of the acetate moiety, rendering the eugenol (Koeduka et al., 2006; Dexter et al., 2007; Aragüez et al., 2013). In contrast, in petunia, PhEOBII regulates the expression of a different set of genes involved in the shikimate and phenylpropanoid pathways, such as *CS*, *CM*, *PAL2*, *CFAT*, *IGS*, *BPBT*, and *ODO1* (Spitzer-Rimon et al., 2010). Thus, our results indicate that, in contrast to what is described in petunia, in strawberry receptacle, it seems that FaEOBII directly regulates the expression of both *FaCAD1* and *FaEGS2* genes. This possibility was also supported by the fact that FaEOBII can transactivate in vivo the *FaCAD1* promoter (Fig. 9) and can also specifically bind to MBSII boxes, MYB cis-regulatory sequences, which are present in the promoters of those genes regulated by R2R3 MYB TFs (Fig. 10). Additionally, the statistically significant decrease of eugenol (Fig. 8A) in strawberry fruit receptacles where the expression of *FaEOBII* was silenced (Fig. 8B) confirms the direct connection between the functional activity of this TF and eugenol biosynthesis. However, as stated previously, the lack of a strong correlation between the percentage of gene silencing and eugenol content reduction could be due to the fact that the amount of transcript (arbitrary units of intensity [a.u.i.]) corresponding to *FaEGS2* in ripening receptacle transcriptome is very high (*FaEGS2-gene25260*, a.u.i. = 10,732.21). The amount of eugenol quantified in pFRN-*FaEOBII* fruits (Fig. 8A) could be explained by two reasons. First, the expression of *FaEGS2* was not reduced sufficiently (Fig. 8B) or the residual *FaEGS2* transcript in the fruit could be translated into a sufficient amount of enzyme capable of catalyzing the production of eugenol. Second, the half-life



**Figure 11.** qRT-PCR analysis of *FaMYB10* and *FaEOBII* transcript levels and eugenol and anthocyanin content determination in transgenic agroinfiltrated fruits injected with the corresponding RNAi construct. Statistical significance with respect to the control simple was determined by Student's *t* test: \*,  $P < 0.05$ ; and \*\*\*,  $P < 0.001$  ( $n = 10$ ).

of the enzyme may be high enough so that the detected eugenol was indeed catalyzed by FaEGS2 enzyme in the fruit prior to the silencing of *FaEOBII*.

Furthermore, to confirm the regulatory role of FaEOBII in the biosynthesis of eugenol, qRT-PCR analysis was carried out in fruit receptacles overexpressing the gene *FaEOBII*. The results showed not only an increase in the expression of *FaEOBII* and *FaEGS2* genes (Fig. 8D) but also a higher eugenol content with respect to control fruits (Fig. 8C). Although the increase of eugenol is significant in fruits overexpressing *FaEOBII* with respect to control fruits (Fig. 8C), it is difficult to detect higher increases. It is necessary to consider that, in control fruits, the expression of *FaEOBII* and *FaEGS2* in the transcriptome of red-ripened receptacles is already very high (*FaEOBII-gene28435*, a.u.i. = 3,198.538; *FaEGS2-gene25260*, a.u.i. = 10,732.21), as is eugenol content, so the production system of eugenol may be saturated in fruits overexpressing *FaEOBII* and greater increases of eugenol may not be detected.

#### *FvEGS2* and *FaEGS2* Have Different Expression Patterns That Correlate with MBSII cis-Regulatory Elements in Their Promoters

Gene expression analyses of *EGS2* and *EOBII* were performed by qRT-PCR in ripe *F. vesca* and *F. ananassa* fruits. Significant differences in *EGS2* expression were observed between the two species, up to more than 20-fold in *F. vesca* than in *F. ananassa*, whereas *EOBII* expression values were constant in both species (Fig. 4A). Bioinformatic analysis of promoter sequences

located along the *EGS2* promoter identified several MBSII boxes recognized by FaEOBII, containing more MBSII boxes of the *FvEGS2* promoter than the *FaEGS2* promoter (Supplemental Fig. S4). These results suggest that higher expression levels of *EGS2* and eugenol content in *F. vesca* than in *F. ananassa* (Fig. 4) could be the result of a higher degree of *FvEGS2* promoter activation by FaEOBII due to the presence of a greater number of MBS regulatory boxes (Supplemental Fig. S4). Nevertheless, results in *F. vesca* may also indicate the presence of other TFs that regulate, synergistically with EOBII, *EGS2* expression.

#### *FaEOBII* Expression Is Ripening Related and Correlates with Eugenol Production in Different Strawberry Plant Tissues

Eugenol is the main phenylpropanoid volatile compound that contributes to strawberry aroma (Pyysalo et al., 1979; Ulrich et al., 1995, 2007; Zorrilla-Fontanesi et al., 2012). In ripe strawberry fruits, eugenol is biosynthesized by the action of FaEGS2, whose coding gene has a ripening-related expression pattern and presents its maximum expression at the red-ripe stage (Aragüez et al., 2013). Besides, the expression of *FaEGS2* was specific to ripe fruit receptacles (Aragüez et al., 2013).

The expression pattern of the *FaEOBII* gene was ripening related and mainly confined to fruit receptacles, although a high expression level in petals was found (Fig. 2). Interestingly, the expression pattern of *FaEOBII* was similar to that of *FaCAD1* and *FaEGS2* (Fig. 3A) and other ripening-related genes that are responsible for the organoleptic properties of the strawberry fruit (Medina-Escobar

et al., 1997; Moyano et al., 1998; Trainotti et al., 1999; Blanco-Portales et al., 2002, 2004; Benítez-Burraco et al., 2003; Raab et al., 2006; Griesser et al., 2008; Cumplido-Laso et al., 2012; Molina-Hidalgo et al., 2013; Medina-Puche et al., 2014). Besides, the maximum *FaEOBII* gene expression as well as *FaCAD1* and *FaEGS2* expression were found in those tissues, such as red-ripened receptacles or petals, where eugenol content was highest (Fig. 3B). This indicates that, as in the case of *PhEOBII* in petunia petals (Spitzer-Rimon et al., 2010; Van Moerkercke et al., 2011), *FaEOBII* could regulate the expression of those structural genes that are responsible for the production of eugenol in both ripe fruit receptacles and petals.

### In Ripe Receptacles, Eugenol Production Is Regulated by the Interaction between FaMYB10 and FaEOBII

Recently, the key role played by *FaMYB10* in the regulation of those genes that are part of the flavonoid/phenylpropanoid metabolism has been functionally characterized (Medina-Puche et al., 2014). In that work, after silencing *FaMYB10* expression, a down-regulation of the expression of *FaCAD1*, *FaEGS2*, and *FaEOBII* was observed (Medina-Puche et al., 2014). We have confirmed this result by qRT-PCR, and we also showed that, after *FaMYB10* silencing, there is a concomitant reduction in the receptacle eugenol content (Fig. 11). These results, together with the fact that both TFs share the same expression pattern, support the fact that the expression of *FaEOBII* is under the control of *FaMYB10* during the fruit-ripening process. This regulatory network is quite different from the one observed in petunia petals (Spitzer-Rimon et al., 2010; Van Moerkercke et al., 2011). In petunia, the regulation of the production of hydroxycinnamoyl alcohols, phenylpropanes such as eugenol and isoeugenol, as well as other volatile benzenoid compounds is carried out in a concerted manner by three R2R3 MYB TFs named *PhEOBII*, *ODO1*, and *EOBI* (Verdonk et al., 2003, 2005; Schuurink et al., 2006; Colquhoun et al., 2010b, 2011; Spitzer-Rimon et al., 2010, 2012; Colquhoun and Clark, 2011; Van Moerkercke et al., 2011). *PhEOBII* directly regulates the expression of *ODO1* and *EOBI*, which in turn regulate the expression of several structural genes corresponding to the biosynthesis of volatile phenylpropanoids. On the contrary, in ripe strawberry receptacles, *FaMYB10* would activate the expression of *FaEOBII*, which could subsequently trigger both the expression of *FaCAD1* and *FaEGS2* genes and eugenol production. Thus, although the involvement of the *EOBII* TF in the regulation of eugenol production has been maintained in both petunia and strawberry, the regulatory loops involved are clearly different. This may be because of the evolutionary distance between petunia (an asterid) and *F. ananassa* (a rosid).

### *FaEOBII* Expression Is Hormonally Regulated in a Similar Way to That of Other Strawberry Ripening-Related TFs

As in the case of *FaMYB10*, *FaEOBII* gene expression was regulated in an antagonist manner by auxins and

ABA, the two most important hormones that control strawberry developmental and ripening processes (Perkins-Veazie, 1995; Chai et al., 2011; Jia et al., 2011; Medina-Puche et al., 2014). Thus, the decline in the inner contents of auxins in green unripe receptacles gave rise to an increased *FaEOBII* expression (Fig. 5). This expression model has been described for other receptacle-specific and ripening-related genes (Moyano et al., 1998; Blanco-Portales et al., 2002; Raab et al., 2006; Cumplido-Laso et al., 2012; Molina-Hidalgo et al., 2013). On the contrary, when ABA content in the receptacle was diminished by transient silencing of *FaNCED1* expression or inhibition of the corresponding enzyme activity by the inhibitor NDGA, a decrease in *FaEOBII* expression was observed. However, when the concentration of ABA was increased in fruit receptacles as a consequence of subjecting the fruits to water stress, an increase in *FaEOBII* gene expression was observed (Fig. 6). In both climacteric and nonclimacteric fruits, water deficiency generated a rise 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). This dual regulation has also been observed for other genes whose expression is also up-regulated throughout the strawberry fruit-ripening process (Cumplido-Laso et al., 2012; Daminato et al., 2013; Molina-Hidalgo et al., 2013) and molecularly supported both the proposal of Perkins-Veazie (1995) and the findings described by Chai et al. (2011), Jia et al. (2011), and Medina-Puche et al. (2014). This hormonally regulated expression pattern was also similar to that observed for the *FaMYB10* gene (Medina-Puche et al., 2014), and it strengthens the relationship between *FaMYB10* and *FaEOBII*. In this sense, we cannot discount that the hormonal regulation of *FaEOBII* could be indirect as a result of the regulation of *FaEOBII* expression by *FaMYB10*.

## CONCLUSION

In summary, this work presents novel functional data indicating that *FaEOBII* is a hormonal and ripening-related TF that regulates, by a novel regulatory network, key structural genes of the phenylpropanoid pathway that are related to eugenol biosynthesis in the strawberry fruit receptacle.

## MATERIALS AND METHODS

### Plant Material

Strawberry plants (*Fragaria* × *ananassa* 'Camarosa,' an octoploid cultivar, and *Fragaria vesca*, a diploid wild strawberry) were grown under field conditions in Huelva, in southwestern Spain. *F. ananassa* fruits were harvested at different developmental stages: small-sized green fruits (2–3 g), full-sized green fruits (4–7 g), white fruits (5–8 g), full-ripe red fruits (6–10 g), over-ripe fruits (6–10 g), and senescent fruits (6–10 g). Selected overripe fruits were harder and dark redder than red ones, while senescent fruits were softer. Other tissues were harvested as well, such as stolons, roots, crowns, flowers, petals, and expanding leaves. All tissues were immediately frozen in liquid nitrogen and stored at –80°C. *Nicotiana benthamiana* and strawberry

(*F. ananassa* 'Elsanta') plants used for agroinfiltration were grown in plant chambers at 25°C, 10,000 lux, and 80% humidity and afterward were held in a greenhouse.

### Auxin Treatment

Achenes of two sets of 50 full-sized green fruits (G3) each were carefully removed from their receptacles. One set of deached G3 fruits was treated with IAA in lanolin paste (1 mL) containing 1 mM IAA in 1% (w/v) dimethyl sulfoxide. The other set of G3 deached fruits (control group) was treated with the same paste but without IAA. Auxin treatments, sample collection, and analysis were performed according to Medina-Puche et al. (2014).

### Water Stress Treatment

Fruits at the green to white stages from *F. ananassa* 'Elsanta' were used in this experiment. For water stress treatments, fruit pedicels were kept outdoors, whereas those of control fruits were immersed in Murashige and Skoog medium with Suc (renewed every 2 d). The stress treatment condition and sample analysis followed the instructions of Medina-Puche et al. (2014).

### NDGA Treatment

NDGA is an ideal inhibitor of the NCED enzyme and was used to block ABA biosynthesis (Creelman et al., 1992). Strawberry fruits ('Elsanta') at mature green stages were used in this study. The treatment was performed in accordance with Medina-Puche et al. (2014). These samples were used for ABA content measurement and relative expression of the *FaEOBII* gene.

### Preparation of Deuterated ABA and ABA Extraction Procedure

Deuterated ABA was used as an internal standard. Both the deuterated ABA preparation and ABA extraction from strawberry samples followed the instructions of Medina-Puche et al. (2014).

### HPLC-Mass Spectrometry Conditions

In order to determine the ABA amount in strawberry fruits, we used an HPLC-mass spectrometry system (Varian 1200L Triple Quadrupole) with a column (150- × 2.1-mm-i.d. Phenomenex C<sub>18</sub> with a 3-μm particle). The conditions and procedure used for the analysis were those described by Medina-Puche et al. (2014).

### Solid-Phase Microextraction of Eugenol

Eugenol was analyzed in *F. ananassa* 'Elsanta' fruits infiltrated with *Agrobacterium tumefaciens* carrying pFRN, pFRN-*FaEOBII*, pK7WG2.0, or pK7WG2-*FaEOBII* and *F. ananassa* 'Camarosa' and *F. vesca* fruits at different developmental stages. Ten strawberry fruits were harvested per sample, and three samples were measured on each occasion. Fruits were ground to a fine powder under liquid nitrogen. Saturated NaCl (300 μL) was added to 1 g, 200 mg, or 10 mg of powdered *F. ananassa*, *F. vesca* white fruits, or *F. vesca* red fruits, respectively. β-Caryophyllene (3 ng) was added as an internal standard. Solid-phase microextraction was performed on a 7890A GC system (Agilent; <http://www.home.agilent.com/>) coupled to a 7200A QTOF-MS device (Agilent). Samples were incubated for 10 min at 50°C, after which volatiles were extracted on a 100-μm PDMS fiber (Supelco; [www.sigmaaldrich.com/](http://www.sigmaaldrich.com/)) for 30 min at 50°C. Extracted compounds were desorbed for 1 min at 250°C in the injector port in splitless injection mode. Volatiles were separated on an HP5MS column (5% phenyl methyl silox, 30 m × 250 μm × 0.25 μm), with a constant helium flow of 1.2 mL min<sup>-1</sup>. Oven temperature was set to 40°C for 3 min, ramped at 5°C min<sup>-1</sup> to 250°C, and held at 250°C for 5 min. Twenty mass spectra were recorded every second (mass-to-charge ratio 30–500) at an ionization energy of 70 eV. Eugenol was measured in commercial strawberry, and no eugenol was detected above the detection limits of the system. Therefore, a calibration curve of eugenol (Acros Organics) carried out in extracts of these strawberries lacking eugenol was used to identify and quantify it. Statistical significance was tested with one-way ANOVA and an LSD post hoc test or with Student's *t* test using SPSS software.

### Total Anthocyanidin Analysis

Anthocyanidin extraction was performed following the instructions of Medina-Puche et al. (2014). The anthocyanidin presence was measured at 515 nm. The amount of anthocyanins was calculated by using  $E_{\text{molar}} = 36,000 \text{ L mol}^{-1} \text{ cm}^{-1}$  (Woodward, 1972; Bustamante et al., 2009). Three replicates were performed for each analyzed treatment.

### RNA Isolation

Total RNA was isolated from independent pools of strawberry fruits at different growth and ripening stages and from vegetative tissues in accordance with Asif et al. (2000). Achenes were always removed from fruits, and only receptacle RNA was extracted and purified. The total RNA obtained was treated with DNase I (RNase free; Invitrogen), following the manufacturer's instructions, and further purified using the RNeasy Mini Kit (Qiagen). RNA samples were considered DNA free when no amplicons corresponding to those analyzed genes were observed using RNA as template in a standard PCR.

### Microarray Generation and Analysis

We compared the cDNA sequences contained in Kevin Folta's libraries (Shulaev et al., 2011) with our own previously obtained cDNA libraries (Bombarely et al., 2010). The percentage identity between the cDNA sequences from *F. ananassa* and those from *F. vesca* was always over 98.6% and similar to that found previously (Bombarely et al., 2010). Therefore, we decided to generate a custom-made oligonucleotide-based (60-mer length) microarray platform containing a total of 34,187 singletons corresponding to those sequences published in the Strawberry Genome Project (<https://strawberry.plantandfood.co.nz/>).

For each sequence, four different oligonucleotides were printed per block, and four blocks were printed per data set. Samples of total RNA were DNase I treated and afterward purified with Qiagen columns, in accordance with the manufacturer's conditions. Sample labeling (Cy3), hybridization, 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 of total receptacle RNA was processed using the Roche cDNA Synthesis System, and cDNAs were purified using High Pure PCR Product Purification following recommendations. Three biological replicates were performed, obtaining three independent cDNA populations. Each cDNA sample (1 μg) was random primer labeled with Cy3-nonamers using NimbleGen One-Color DNA Labeling Kits, in accordance with Roche NimbleGen's standard protocol. Using random assignment, each Cy3-labeled cDNA sample was applied to custom-made strawberry 12 × 135K array formats (each slide containing 12 independent arrays, each with 140,856 probes covering 34,187 genes, four probes per target gene). The array was then hybridized for 16 h at 42°C, washed, dried, and scanned at a 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 scanned images and to perform robust multiarray analyses to generate raw gene expression values.

Differential expression analysis was performed using the Array Star software (DNASTAR). Moderated Student's *t* test and false discovery rate (Benjamini and Hochberg, 1995) for multiple testing corrections were used at  $P < 0.01$  to statistically identify significant differences.

### Validation of Microarray Data and Expression Analysis by qRT-PCR

Expression analyses of *FaEOBII* (KM099230) and the genes herein analyzed and studied in all physiological conditions were performed by qRT-PCR using the iCycler system (Bio-Rad), as described previously (Benítez-Burraco et al., 2003). First strand cDNA was obtained from 2 μg of total RNA using the iScript kit (Bio-Rad) following the manufacturer's instructions. Supplemental Table S5 depicts the primer sequences used for quantitative amplification. Each reaction was performed at least in triplicate, and the corresponding C<sub>t</sub> values were normalized using the C<sub>t</sub> 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; Molina-Hidalgo et al., 2013). All these values were then used to determine the relative increase or decrease of *FaEOBII* expression in the samples as compared with those in the control, in accordance with Pedersen (2001).

The *interspacer 26S-18S* gene (primers 5'-ACCGTTGATTCGCACAATTGGTCATCG-3' and 5'-TACTGCGGGTCGGCAATCGGACG-3') was selected as a control gene owing to its constitutive expression throughout all of the different tested experimental conditions (Supplemental Table S6). The efficiency of each particular qRT-PCR and the melting curves of the products were also analyzed to ensure the existence of a single amplification peak corresponding to a unique molecular species (melting temperature was 87°C for the *FaEOBII* gene and 91.5°C for the *interspacer 26S-18S* gene).

## Cloning Full-Length cDNA of FaEOBII and Sequence Analysis

We used the resources of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) and the European Bioinformatics Institute server (<http://www.ebi.ac.uk/>) for the *in silico* study of the *FaEOBII* gene sequence. To compare the nucleotide *FaEOBII* sequence against databases, we used the National Center for Biotechnology Information BLASTN program as an alignment tool. Multiple sequence alignment and phylogenetic tree construction were performed with the European Bioinformatics Institute ClustalW2 program or the MegAlign program (from the Lasergene suite) as well as the FigTree program (<http://tree.bio.ed.ac.uk/software/figtree/>). Prediction of domains and functional sites was performed with the InterProScan database (version 4.8; [www.ebi.ac.uk/Tools/pfa/ipscan/](http://www.ebi.ac.uk/Tools/pfa/ipscan/)), and prediction of cellular location was performed with the WoLF PSORT program ([http://www.genscript.com/psort/wolf\\_psort.html](http://www.genscript.com/psort/wolf_psort.html)). BLASTN has also been used to determine gene location in the *F. vesca* genome in the Genome Database for Rosaceae data bank (<http://www.rosaceae.org>).

## Generation of FaEOBII-RNAi and FaEOBII Overexpression Constructs and Transfection of Strawberry Fruit by Agroinfiltration

The *FaEOBII* gene was cloned into pFRN binary vector (Supplemental Fig. S6; courtesy of Marten Denekamp, Department of Molecular Cell Biology, University of Utrecht) using Gateway technology (Invitrogen). A 448-bp conserved region from the *FaEOBII* cDNA was PCR amplified and used in the silencing construct, using forward 5'-AGGGCCGTGGACCATGGAAGAGG-3' and reverse 5'-CGCCGTCGGGGCGCATAGAGG-3' primers. The resulting fragment was cloned into pCR8/GW/TOPO (Invitrogen) and subsequently transferred to the Gateway pFRN vector using LR Clonase (Invitrogen). The RNAi construct generated (pFRN-*FaEOBII*) was tested by sequencing prior to strawberry plant transformation.

The 624-bp coding sequence of strawberry *FaEOBII* was amplified from *F. ananassa* cDNA using the oligonucleotides attB1-EOBII-fw (5'-GGGGACAAAGTTGTACAAAAAGCAGGCTTAACAATGGATAAAAAACCATGCAATTCATC-3') and attB2-EOBII-rv (5'-GGGGACCACITTTGTACAAAGAAAGCTGGGCTTACTCTCCATTTAGTAAGTGCATAGACC-3'), cloned into the pDONR221 (Invitrogen) plasmid by the BP Gateway reaction (Invitrogen), and subsequently transferred to the Gateway pK7WG2.0 vector using LR Clonase (Invitrogen). The overexpression construct generated (OE-*FaEOBII*) was tested by sequencing prior to strawberry plant transformation.

The *A. tumefaciens* strain AGL0 (Lazo et al., 1991) containing the pFRN-*FaEOBII* or empty pFRN vector and pK7WG2-*FaEOBII* or empty pK7WG2 vector was used to inject entire strawberry fruits (*F. ananassa* 'Elsanta'), according to Hoffmann et al. (2006). Overexpression of *FaEOBII* was carried out by the coinfiltration of *A. tumefaciens* harboring the silencing suppressor pTBSV-p19 (Voinnet et al., 2003). A total of 15 to 25 strawberry plants and 30 to 40 agroinjected fruits were inoculated and analyzed, respectively. The same protocol was followed to generate the *FaNCED1*-RNAi and *FaMYB10*-RNAi constructs and to silence *FaNCED1* and *FaMYB10* expression in strawberry fruits. Forward and reverse primers used for *FaNCED1* were 5'-CGGTGCGCCAGCTACGCAT-3' and 5'-GTGGGGCCGAGAGGGAT-3', while those for *FaMYB10* were 5'-AGATGACTAGATGATTGCTGCGC-3' and 5'-TGC-CGGACGATTGCCAGGAAG-3', respectively (Medina-Puche et al., 2014).

In all cases, the silencing or overexpression percentages were determined by comparing the amounts of *FaEOBII*, *FaNCED1*, or *FaMYB10* transcripts in RNAi and overexpression agroinjected fruits against those observed in empty pFRN or pK7WG2 vector agroinfiltrated fruits, respectively.

## Subcellular Location Analysis

The 624-bp coding sequence of strawberry *FaEOBII* was amplified from *F. ananassa* cDNA using the oligonucleotides attB1-EOBII-fw

(5'-GGGGACAAGTTGTACAAAAAGCAGGCTTAACAATGGATAAAAAACCATGCAATTCATC-3') and attB2-EOBII-rv (5'-GGGGACCACITTTGTACAAAGAAAGCTGGGCTTACTCTCCATTTAGTAAGTGCATAGACC-3') and cloned into the pDONR221 (Invitrogen) plasmid by the BP Gateway reaction (Invitrogen). *FaEOBII* was fused in frame with GFP at the N-terminal position by LR Gateway recombination (Invitrogen) in the destination vector pK7WGF2 (Karimi et al., 2002), resulting in the 35S::GFP::FaEOBII fusion. The generated GFP construct (pK7WGF2-*FaEOBII*) was tested through sequencing prior to *N. benthamiana* leaf transformation.

Agroinfiltration experiments were performed on *N. benthamiana* leaves. The *A. tumefaciens* strain GV3101 (Weigel and Glazebrook, 2006) containing pK7WGF2-*FaEOBII*, the empty pK7WGF2, and the silencing suppressor pTBSV-p19 (Voinnet et al., 2003) was grown at 28°C in Luria-Bertani medium with appropriate antibiotics. Cells were harvested and resuspended in an infiltration buffer (10 mM MgSO<sub>4</sub>, 10 mM MES, and 1 mM acetosyringone). This step was repeated once, and the concentration of bacterial suspension was measured by spectroscopy (optical density at 600 nm) and adjusted to a final concentration of 0.5 to 0.8. Then, the cells carrying the pK7WGF2-*FaEOBII* and the empty pK7WGF2 constructs were mixed with cells containing the p19 vector pairwise and left at room temperature for 2 to 4 h in darkness. Bacterial suspensions were syringe infiltrated through the abaxial surface of the leaves, and material was collected after 4 d. To locate nuclei precisely, *N. benthamiana* leaf tissues were incubated with DAPI. Leaves were observed by epifluorescence microscopy (LSM 5 EXCITER; Carl Zeiss), with excitation/emission settings of 488/500 to 515 nm for GFP and 405/449 to 461 nm for DAPI.

## Protein Purification and Determination of FaEOBII DNA-Binding Specificity

Translational fusion of *FaEOBII* to Maltose Binding Protein was obtained by cloning its corresponding cDNA into the pMAL-c2 vector (New England Biolabs) using Gateway technology. Donor template was obtained through PCR amplification of *FaEOBII* with specific oligonucleotides attB1-EOBII-fw (5'-GGGGACAAGTTGTACAAAAAGCAGGCTTAACAATGGATAAAAAACCATGCAATTCATC-3') and attB2-EOBII-rv (5'-GGGGACCACITTTGTACAAAGAAAGCTGGGCTTACTCTCCATTTAGTAAGTGCATAGACC-3'). The recombinant insert was verified by sequencing, and the plasmid was introduced into *Escherichia coli* BL-21. The expression and purification of recombinant protein were also described for the pMAL purification system (New England Biolabs). *FaEOBII* DNA-binding specificity was determined using protein-binding microarrays (PBM11) using soluble protein extracts from an induced *E. coli* culture, as described by Godoy et al. (2011).

## Isolation and *In Silico* Analysis of the CAD Promoter

The promoter region of *CAD* was identified from the strawberry genome database (<http://www.strawberry.org>; Shulaev et al., 2011), and PLACE (<http://www.dna.affrc.go.jp>) was used to detect cis-acting regulatory DNA elements in the *CAD* promoter (Higo et al., 1999). For further analysis, a 2,638-bp fragment of the promoter region, including the 5' untranslated region, was isolated from genomic DNA using primers pCAD-up (5'-ACTCCGACGTTTGTGAGAGA-3') and pCAD-low (5'-TCTCGATCTGTGGCAGATTCTT-3') and following the manufacturer's instructions for the Phire Plant Direct PCR Kit (Thermo Scientific). PCR products were purified and cloned into the pCR8/GW/TOPO entry vector (Invitrogen) by TOPOTA Cloning (Invitrogen). pCAD1 was fused with GUS by LR Gateway recombination (Invitrogen) in destination vector pKGWFS7 (Karimi et al., 2002), resulting in pCAD1::GUS fusion. The generated GUS construct (pKGWFS7-pCAD1) was sequence verified prior to *N. benthamiana* leaf transformation.

## Transient Transactivation Assay in *N. benthamiana* Leaves

*A. tumefaciens* GV3101 (pMP90) cultures harboring reporter or effector constructs were grown overnight and diluted in infiltration buffer (50 mM MES, pH 5.8, 0.5% [w/v] Glc, 2 mM Na<sub>3</sub>PO<sub>4</sub>, and 100 μM acetosyringone) to an optical density at 600 nm of 0.3 prior to infiltration in *N. benthamiana* leaves. Combinations of reporter and effector constructs were coinfiltrated in leaves of glasshouse-grown *N. benthamiana* plants and incubated for 48 h. Infiltration regions were marked, and five leaf discs were pooled per sample. Six leaves on two plants were infiltrated for each effector/reporter combination (*n* = 6), and the experiment was repeated twice. To enable normalization, leaves were coinfiltrated with a CaMV 35S::LUCIFERASE (LUC)-harboring *A. tumefaciens*.



Tissues were homogenized in liquid N<sub>2</sub>, and proteins were extracted from 50 mg of tissue in 150  $\mu$ L of cold luciferase cell culture lysis reagent (25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 2 M 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 1% [v/v] Triton X-100, and 10% [v/v] glycerol [Promega; <http://www.promega.com>]) supplemented with a complete protease inhibitor (Roche; <http://www.roche.com>). Samples were vortexed for 30 s and centrifuged at 4°C for 30 min at 13,000g. GUS activity of crude extracts was determined spectrophotometrically using 1 mM 4-methylumbelliferyl-D-glucuronide in cell culture lysis reagent supplemented with 10 mM  $\beta$ -mercaptoethanol as substrate after a 30-min incubation period at 37°C. Measurements were performed in a FluoroCount Microplate Fluorometer (Packard BioScience; <http://www.packardinstrument.com>) using a 360-nm excitation filter and a 460-nm emission filter. For LUC measurements, 20  $\mu$ L of extract was analyzed in 80  $\mu$ L of luciferase assay buffer (20 mM Tricine, 2.67 mM MgSO<sub>4</sub>, 0.1 mM EDTA, 33 mM dithiothreitol, 270  $\mu$ M CoA, 530  $\mu$ M ATP, and 470  $\mu$ M D-luciferin, pH 7.8; Leeuwen et al., 2000) and measured in a FluoroCount Microplate Fluorometer (Packard BioScience) using a 560-nm emission filter (Van Moerkercke et al., 2011).

For promoter analysis in *N. benthamiana* leaves and the transactivation assays, the *pODO1*:GUS reporter construct was used as a control and *pCAD1*:GUS was used as a test. GUS and LUC activity were determined as described by Van Moerkercke et al. (2011). For the transactivation assays, 35S:*FaEOBII* was used as the effector construct. As a control, the previously described constructs 35S:*PhEOBII*, 35S:*AN2*, and 35S:*LUC* (Verdonk et al., 2005; Van Moerkercke et al., 2011) were used.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers FaEOBII (KM099230), FaMYB10 (EU155162), PhEOBII (EU360893), FvEOBII (XP004304632), FaEGS1 (KF562264), FaEGS2 (KF562266), FaCAD1 (U63534), and FaNCED1 (HQ290318).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Screenshots corresponding to the results of bioinformatic predictions.

**Supplemental Figure S2.** Sequence alignment of R2R3-MYB proteins.

**Supplemental Figure S3.** Phylogenetic tree of 206 MYB TFs.

**Supplemental Figure S4.** Schematic diagram of the *EGS2* promoter from *F. ananassa* and *F. vesca*.

**Supplemental Figure S5.** Overview of the phenotypes of fruits analyzed in studies of gene expression regulated by ABA.

**Supplemental Figure S6.** Structure of the binary vector pFRN.

**Supplemental Table S1.** Subgroup classification of 61 plant MYB sequences examined in this study and additional information.

**Supplemental Table S2.** MYB TFs from strawberry.

**Supplemental Table S3.** Total microarray data from a transcriptomic comparison between transgenic transiently silenced *FaEOBII* fruits and control fruits.

**Supplemental Table S4.** Microarray and qRT-PCR results of the differentially regulated genes after silencing *FaEOBII*.

**Supplemental Table S5.** Primer sequences used for qRT-PCR.

**Supplemental Table S6.** Gene expression values of different housekeeping genes obtained by qRT-PCR analysis.

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