

RESEARCH PAPER

A SHATTERPROOF-like gene controls ripening in non-climacteric strawberries, and auxin and abscisic acid antagonistically affect its expression

Margherita Daminato¹, Flavia Guzzo² and Giorgio Casadoro^{1,3,*}

¹ Department of Biology, University of Padua, Via G. Colombo, 3, I-35131 Padua, Italy

² Department of Biotechnology, University of Verona, Strada Le Grazie 15, Ca' Vignal 1, I-37134 Verona, Italy

³ Botanical Garden, University of Padua, Via Orto Botanico, 15, I-35123 Padua, Italy

*To whom correspondence should be addressed. E-mail: giorgio.casadoro@unipd.it, casadoro@mail.bio.unipd.it

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Abstract

Strawberries (*Fragaria×ananassa*) are false fruits the ripening of which follows the non-climacteric pathway. The role played by a C-type MADS-box gene [*SHATTERPROOF*-like (*FaSHP*)] in the ripening of strawberries has been studied by transiently modifying gene expression through either over-expression or RNA-interference-mediated down-regulation. The altered expression of the *FaSHP* gene caused a change in the time taken by the over-expressing and the down-regulated fruits to attain the pink stage, which was slightly shorter and much longer, respectively, compared to controls. In parallel with the modified ripening times, the metabolome components and the expression of ripening-related genes also appeared different in the transiently modified fruits. Differences in the response time of the analysed genes suggest that *FaSHP* can control the expression of ripening genes either directly or indirectly through other transcription factor-encoding genes. Because fleshy strawberries are false fruits these results indicate that C-type MADS-box genes like *SHATTERPROOF* may act as modulators of ripening in fleshy fruit-like structures independently of their anatomical origin. Treatment of strawberries with either auxin or abscisic acid had antagonistic impacts on both the expression of *FaSHP* and the expression of ripening-related genes and metabolome components.

Key words: Abscisic acid, auxin, *Fragaria×ananassa*, fruit metabolome, fruit ripening, MADS-box gene, non-climacteric fruit, *SHATTERPROOF*-like gene, strawberry.

Introduction

The evolution of seeds brought about the necessity to disperse them into the environment. A very successful tool for seed dispersal is represented by fleshy fruits, which attract frugivorous animals by means of pleasant colours and aromas, thus imposing on them the task of dispersing the seeds via their faeces. Besides being pleasant to eat, fleshy fruits also contain numerous secondary metabolites with positive effects on human health that are being unravelled by researchers around the world. However, fleshy fruits are perishable commodities

due to their limited post-harvest life; therefore the idea of extending their life has led to the search for possible factors controlling the process of ripening (Seymour *et al.*, 2012).

Since their discovery, plant hormones have been assayed for their activity on fleshy fruit development and ripening. Ethylene has striking effects on the ripening of climacteric fruits, which exhibit a respiratory climacteric peak prior to the start of ripening. Interestingly, such fruits also show a burst of ethylene production concomitant with the

Abbreviations: ABA, abscisic acid; EST, expressed sequence tag; FW, forward; HPLC-ESI-MS, high-performance liquid chromatography-electrospray ionization-mass spectrometry; NAA, 1-naphthalene acetic acid; OPLS, orthogonal projections to latent structures; RT, reverse transcription; RV, reverse.

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respiratory climacteric, whereas other fruits show neither type of climacteric rise and were thus named non-climacteric fruits (Alexander and Grierson, 2002; Klee and Giovannoni, 2011).

In spite of the economic importance of some non-climacteric fruits (e.g. grape, strawberry, citrus, etc.), ripening was mostly studied in the climacteric ones. In particular, ethylene is not the sole controller of ripening because other important checkpoints are situated upward of ethylene (Klee and Giovannoni, 2011). The latter finding was made possible by the molecular characterization of some tomato (*Solanum lycopersicon*) mutants, such as ripening inhibitor (*rin*; Vrebalov *et al.*, 2002), non-ripening (*nor*; Giovannoni, 2004), and colorless non-ripening (*cnr*; Manning *et al.*, 2006). Their fruits, besides being unable to produce any climacteric ethylene, are also unable to ripen when treated with the hormone. In all cases the mutation involved genes coding for transcription factors that were not expressed with their normal pattern.

In particular, the *RIN* gene encodes a MADS-box transcription factor (Vrebalov *et al.*, 2002) and some of the latter transcription factors are known to be involved in the determination of flower components. Hence, as already seen for *Arabidopsis* dry fruit, it appears that at least some of the genes involved in flower development are recruited later on for the subsequent transformation of the ovary into a fleshy fruit. It was thus seen that MADS-box genes are involved in the development and ripening of fruits in peach (*Prunus persica*; Tadiello *et al.*, 2009), tomato (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009), banana (*Musa acuminata*; Elitzur *et al.*, 2010), and oil palm (*Elaeis guineensis*; Tranbarger *et al.*, 2011) but also in the non-climacteric bilberry (*Vaccinium myrtillus*; Jaakola *et al.*, 2010) and strawberry (*Fragaria×ananassa*; Seymour *et al.*, 2011). Although non-climacteric fruits have always been considered a group apart, the involvement of MADS-box genes in their ripening suggests that at least some aspects of this process might be shared by all fruit types.

Strawberry is not ovary-derived and is therefore a false fruit, yet many biochemical and molecular studies have demonstrated that the ripening syndrome in strawberry is similar to that of botanical fruits (Aharoni and O'Connell, 2002; Fait *et al.*, 2008), albeit with specific differences that distinguish one fruit from another. The false strawberry fruit is comparable to the fleshy fruit-like structures produced by some Gymnosperms in which C-type MADS-box genes have been seen to be involved in development and ripening (Lovisetto *et al.*, 2012); therefore, it appeared interesting to study the role played by C-type MADS-box genes in the ripening of strawberries. However, flower-producing plants normally have at least two different C-type MADS-box genes: one gene is included in the *AGAMOUS/FARINELLI* clade while the other belongs to the *PLENA/SHATTERPROOF* subgroup (*euAGAMOUS* and *PLENA* subgroups, respectively, according to Kramer *et al.*, 2004).

A role in fruit development and ripening has recently been shown for the genes belonging to the *PLENA/SHATTERPROOF* subgroup in climacteric peaches (Tadiello *et al.*, 2009) and tomatoes (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009); hence it was deemed of interest to study

the regulatory role played by a gene belonging to the same subgroup in the non-climacteric false fruit of the strawberry. To such a purpose the expression of a *SHATTERPROOF*-like gene was altered through either down-regulation or over-expression. It has thus been shown that in strawberry this gene plays a role as a modulator of the ripening syndrome. Moreover, its expression appeared to be antagonistically regulated by the hormones auxin and abscisic acid (ABA).

Materials and methods

Plant material

Strawberry plants (*Fragaria×ananassa* Duch. cv. Elsanta) were grown under standard conditions at 25 °C and a 16-h photoperiod in a greenhouse at the Department of Biology, University of Padua, Italy. Fruits were harvested at different developmental stages: small green (G1), large green [G2(1)], large green with enlarged achenes [G2(2)], white with green achenes (W1), white with brown achenes (W2), pink (P), and red (R). Fully expanded leaves and flowers at anthesis were also harvested. All tissues and fruit samples were frozen and stored at -80 °C.

RNA extraction and gene-expression analysis

Total RNA extraction, first-strand cDNA synthesis, and gene-expression analysis were carried out similarly to Lovisetto *et al.* (2012). Primer sequences for the selected genes are listed in the Supplementary Table S3.

Cloning of the *FaSHP* cDNA and promoter

The *F.×ananassa SHATTERPROOF*-like (*FaSHP*) full-length cDNA was isolated by screening a strawberry cDNA library (Trainotti *et al.*, 1999). The screening was made following standard procedures using a probe of 200 bp prepared by means of a reverse transcription (RT)-PCR experiment using primers [forward (FW) 5'-GGCACAGCAGCAGCAAGCAAATA-3' and reverse (RV) 5'-AGAGGCGGAATAAATCACCAGACT-3'] designed on expressed sequence tag (EST) sequences (CO380891, EX686940, EX687722) available in public databases. The identity of the isolated cDNA was ascertained by sequencing.

The *FaSHP* gene promoter was obtained by means of RT-PCR carried out using primers (FW 5'-CTCCATGATAATTTGCGAAGATGA-3' and RV 5'-TTGCCAACCAAATGAACGGTGTG-3') designed on the promoter region of the *Fragaria vesca SHP* gene (*FvSHP*). The obtained amplicon was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and was subsequently sequenced.

The sequences can be found in the GenBank database under the following accession numbers: *FaSHP* KC676787, *FaSHP*_promoter KC676788.

DNA sequencing and analysis

DNA sequencing was performed at BMR Genomics (Padua, Italy). Sequence manipulations, analyses, and alignments were performed using the LASERGENE software package (DNASTAR, Madison, WI, USA). Identification of putative *cis*-acting elements in the gene promoter was performed using the following software: PLANTCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>), PLACE (<http://www.dna.affrc.go.jp/PLACE>), and MatInspector (http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html).

Construction of a MADS-Box phylogenetic tree

A set of MADS-box protein sequences downloaded from GenBank (Supplementary Table S4) plus the *FaSHP* sequence obtained in this

work were used to construct a phylogenetic tree that was prepared similarly to that of Lovisetto *et al.* (2012).

Generation of FaSHP over-expression and RNA interference constructs

For the gene over-expression experiments the *FaSHP* coding sequence was cloned in the binary expression vector pBin-AR (Liu *et al.*, 1990). The obtained construct was named pBinAR_FaSHP. The construct for *FaSHP* RNA interference was instead prepared using the pKANNIBAL plasmid (CSIRO, Highett, Australia). A 350-bp fragment corresponding to nucleotides 687–1037 of the *FaSHP* gene was amplified by PCR using forward and reverse primers (FW 5'-AAATCTAGAGAATTCGTCGCGTATGAGCAACCAAT-3' and RV 5'-AAAAAGCTTGGTACCCAAGGAGTGCCTGGCTAGTTC-3'). The *FaSHP* fragment was then inserted into pKANNIBAL vector in inverted repeat configuration according to Wesley *et al.* (2001). Subsequently, the fragment of pKANNIBAL containing the gene-silencing hairpin was subcloned into the binary vector pBINplus (Van Engelen *et al.*, 1995), and the resulting construct was named pBINplus_FaSHPi. A construct to be used as a negative control in the transfection experiments was prepared by cloning the empty pKANNIBAL hairpin into the binary vector pBINplus; this plasmid was named pBINplus_intron (it had a construct carrying an intron placed downstream of the cauliflower mosaic virus 35S promoter). All the prepared binary vectors were introduced into *Agrobacterium tumefaciens* strain AGL0.

Transfection of strawberry fruits by agro-infiltration

Flowers with similar positions on comparable inflorescences of several plants were tagged at anthesis and after 15 days the transfection of fruits that had reached the G2(2) stage was carried out according to Hoffmann *et al.* (2006). These fruits were left attached to the plant and were harvested after reaching the P stage. A total of 30 agro-injected fruits from about 20 plants per each construct were inoculated and analysed.

Auxin and ABA treatments

Three pools, each consisting of 60 strawberry fruits at the W2 stage, were randomly selected on the basis of comparable size and absence of physical damage. The first group was sprayed with a synthetic auxin [2 mM 1-naphthalene acetic acid (NAA) in 0.5% (v/v) Tween 20 (Trainotti *et al.*, 1999)], the second group was sprayed with a solution of 100 mM ABA in 0.5% (v/v) Tween 20 (Chen *et al.*, 2011), and the third group, the control one, was sprayed only with Tween 20 (0.5% v/v). Ten fruits for each group were collected after 0, 2, 4, 6, 12, and 18 h from the beginning of the treatment. Throughout the experiment all the fruits were kept in a growth chamber at 22 °C.

Metabolomics analysis

The powdered frozen fruits were extracted, prepared, and analysed by high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) as described in Supplementary File S1.

To identify the metabolites under *FaSHP* control, an orthogonal projections to latent structures (OPLS) analysis (Simca P+, version 12.0.0.0, Umetrix, Umeå, Sweden) was performed using the 80 HPLC-ESI-MS putatively identified and quantified metabolites as *x* variables and the *FaSHP* mean normalized expression data as *y* variables. In this analysis both untreated and agro-infiltrated fruits were used as controls. The control versus *FaSHP*-down-regulated fruits OPLS model was fully cross-validated with a permutation test, using 200 permutations, and by analysis of variance, while no valid models were obtained using control versus *FaSHP*-up-regulated fruits.

Results

Isolation and characterization of a strawberry *SHP* cDNA

A publicly available cDNA fragment coding for a strawberry *SHATTERPROOF*-like gene was used as a probe to screen a strawberry cDNA library present in our laboratory. The result of this screening was a cDNA of 1134 nucleotides that when sequenced showed that it encoded a full-length *SHP* transcript with untranslated regions at both ends (not shown).

The cDNA was named *F.×ananassa SHATTERPROOF*-like (*FaSHP*) and the sequence was used to construct a phylogenetic tree (Fig. 1A) together with other known C-type sequences belonging to both the *euAGAMOUS* and the *PLENA* clades (Kramer *et al.*, 2004). As expected, *FaSHP* was situated in the *PLENA* clade while the other strawberry C-class sequence (*STAG1*; Aharoni *et al.*, 1999) was located in the *euAGAMOUS* clade. In both cases the strawberry sequences showed a higher similarity with the corresponding sequences of other rosaceous species (Fig. 1A).

In general, *FaSHP* appeared to be more highly expressed than *STAG1* (Fig. 1B). In particular, the expression of *STAG1* increased during fruit growth [stages G2(1)–W1] and declined during the subsequent ripening stages, whereas the expression of *FaSHP* showed a continuous increase during ripening and reached a maximum in pink strawberries where the transcript amount was about 20-fold greater than that of *STAG1*.

Transient down-regulation and over-expression of *FaSHP* in strawberry fruits

The expression of the *FaSHP* gene was transiently altered by either down-regulation or over-expression. To do so, fruits still attached to the plants were infiltrated with a suspension of *A. tumefaciens* cells carrying a construct for either down-regulation through RNA interference or over-expression. Two types of control were used for these experiments, one consisting of untreated fruits and the other consisting of fruits agro-infiltrated with a construct carrying an intron placed downstream of the cauliflower mosaic virus 35S promoter (see Materials and methods). The latter was meant as a control for possible effects caused by the agro-infiltration *per se*.

Since the expression of *FaSHP* started to increase between the large green [G2(2)] and the early white (W1) stages, we decided to agro-infiltrate fruits at the G2(2) stage (see Materials and methods). The rationale for this choice was that by interfering with *FaSHP* expression at such a stage we would be able to either minimize or boost the gene expression increase that normally occurs at the onset of ripening.

The agro-infiltrated fruits were left attached to the plants and harvested at the pink stage; that is, the stage when *FaSHP* normally shows its maximum expression. For all the agro-infiltrated fruits the days necessary to reach this stage were counted. About 30 pink fruits were analysed by real-time PCR for either down-regulation or over-expression of the *FaSHP* gene. The results of such analysis showed that in both cases there were various fruits with significantly altered expression of the gene.

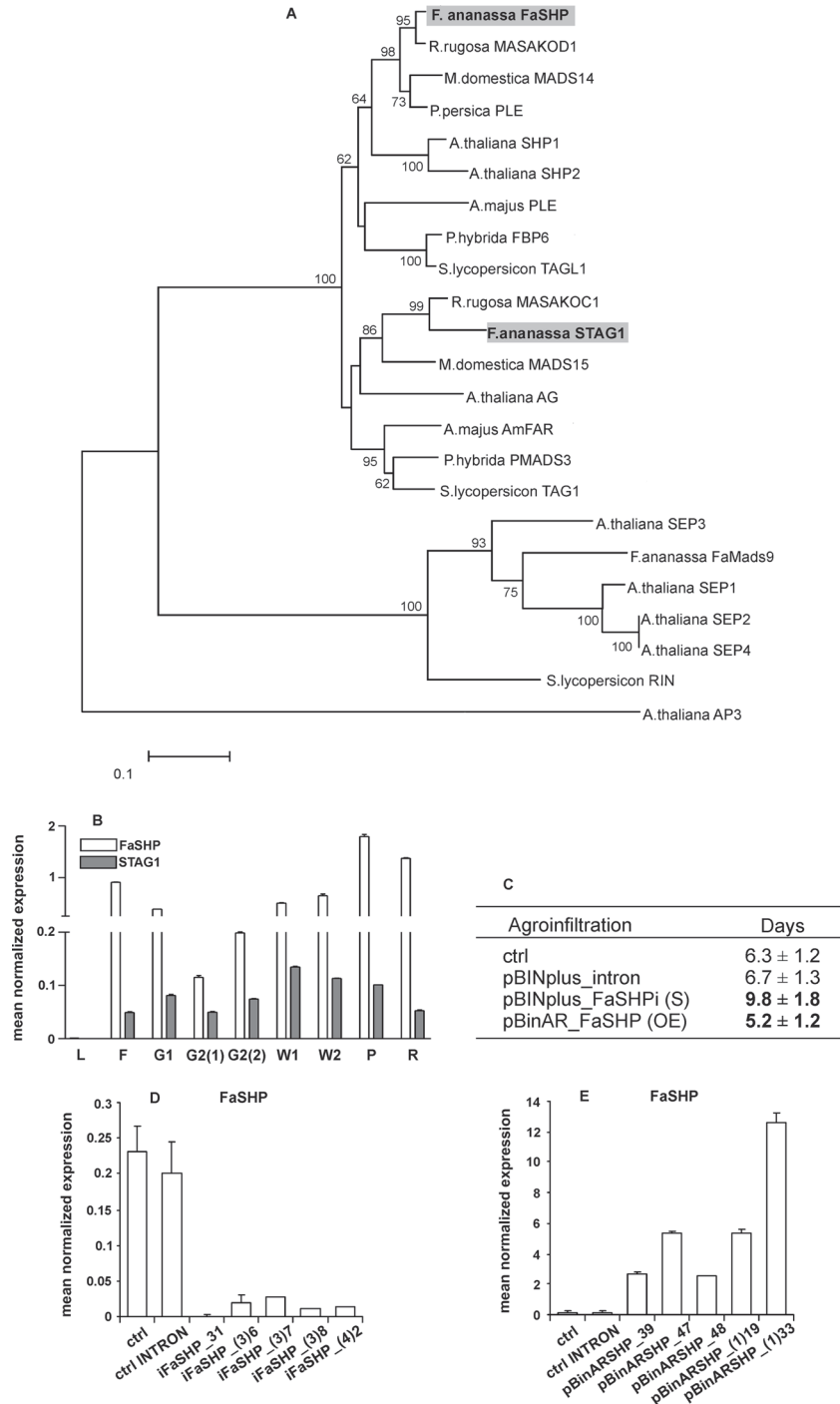


Fig. 1. (A) Phylogenetic tree of eudicot MADS-box proteins. The two C-type strawberry sequences, FaSHP and STAG1 (highlighted by a grey background), appear grouped with the *PLENA* and *euAGAMOUS* clades, respectively. The sequence accession numbers are listed in Supplemental Table S4. (B) Relative expression profiles of the two strawberry C-type MADS-box genes, *FaSHP* and *STAG1*, in leaf (L), flower (F), and fruits at different developmental stages: small green (G1), large green [G2(1)], large green with enlarged achenes [G2(2)], white with green achenes (W1), white with brown achenes (W2), pink (P), and red (R). (C) Number of days necessary to reach the pink stage starting from the G2(2) stage in untreated fruits (ctrl), fruits agro-injected with the control construct (pBINplus_intron), *FaSHP*-silenced fruits (S; pBINplus_FaSHPi), and *FaSHP*-over-expressing fruits (OE; pBinAR_FaSHP). Values are the mean ± standard deviation of five different fruits. Values in bold are significantly different by Student's *t* test from the control agro-injection ($P < 0.05$). (D, E) Relative expression of *FaSHP* in pink fruits that had previously been agro-injected with either (D) the RNA-interference construct (pBINplus_FaSHPi), or (E) the over-expression construct (pBinAR_FaSHP). ctrl and ctrl INTRON values are the means of five independent fruits; the other values are from single fruits. Expression data (means of the normalized expression) were obtained by real-time PCR analyses. Bars are the standard deviations from the means.

For further analyses five independent fruits characterized by either extremely low expression of *FaSHP* (Fig. 1D) or by over-expression of the gene (Fig. 1E) were selected. Pictures of these fruits are available in Supplementary Fig. S1. Interestingly, the altered *FaSHP* expression was accompanied by differences in the number of days necessary to reach the pink stage (Fig. 1C). In both control types the fruits attained a pink colour in about 6 days, while more than 9 days were needed in the case of the down-regulated fruits, and about 5 days were enough for the fruits over-expressing *FaSHP*. As regards the other C-type MADS-box gene of strawberry (i.e. *STAG1*), its expression showed no differences between controls and agro-infiltrated fruits (Fig. 2M).

The general impact on ripening caused by the altered expression of *FaSHP* was then analysed in more detail by studying the expression of selected genes used as markers of various aspects of the ripening syndrome. When fleshy fruits start to ripen their tissues lose firmness and the activity of various cell-wall-modifying enzymes is required for this process (Brummel and Harpster, 2001). Three genes were used as markers of softening: two related to the degradation of pectins [a polygalacturonase gene, *FaPG1* (Quesada *et al.*, 2009) and a pectate lyase gene, *FaPL* (Medina-Escobar *et al.*, 1997)] and one related to the metabolism of hemicellulose [an endo- β -1,4-glucanase gene, *FaEG1* (Trainotti *et al.*, 1999)].

Another important aspect of ripening is the change of colour that is mostly due to the breakdown of chlorophylls and the accumulation of various pigmented molecules. In the case of the

ripe strawberries the red colour is due to anthocyanins, which are phenolic compounds (Perkins-Veazie, 1995). We therefore decided to use two genes as markers for the change of colour: one encoding a phenylalanine ammonia lyase (*FaPAL6*; Pombo *et al.*, 2011), which is the first enzyme in the phenol biosynthetic pathway, and the other encoding a chalcone synthase (*FaCHS*) that has recently been shown to participate in the accumulation of the strawberry red colour (Lunkenbein *et al.*, 2006).

During ripening, strawberries accumulate many secondary metabolites that contribute to their flavour and, in general, for the organoleptic peculiarities of the fruit (Perkins-Veazie, 1995). Furanol is considered to be the main component of the strawberry flavour and a key enzyme for the synthesis of this compound is a quinone oxidoreductase encoded by the *FaQR* gene (Raab *et al.*, 2006), which was thus selected as marker of flavour development. Ripe strawberries are also renowned for their high content of ascorbic acid (Perkins-Veazie, 1995) and a biosynthetic pathway demonstrated to be active during ripening involves the gene *FaGalUR*, encoding a D-galacturonate reductase (Cruz-Rus *et al.*, 2011). Accordingly, this gene was selected as a marker for ascorbic acid accumulation.

All the genes selected as markers were ripening-specific (Supplementary Fig. S2) and the modification of *FaSHP* expression had an impact on their expression profile. The most striking effect on the expression of all the marker genes was observed in fruits with down-regulated *FaSHP* expression (Fig. 2A–2G) because their expression also appeared

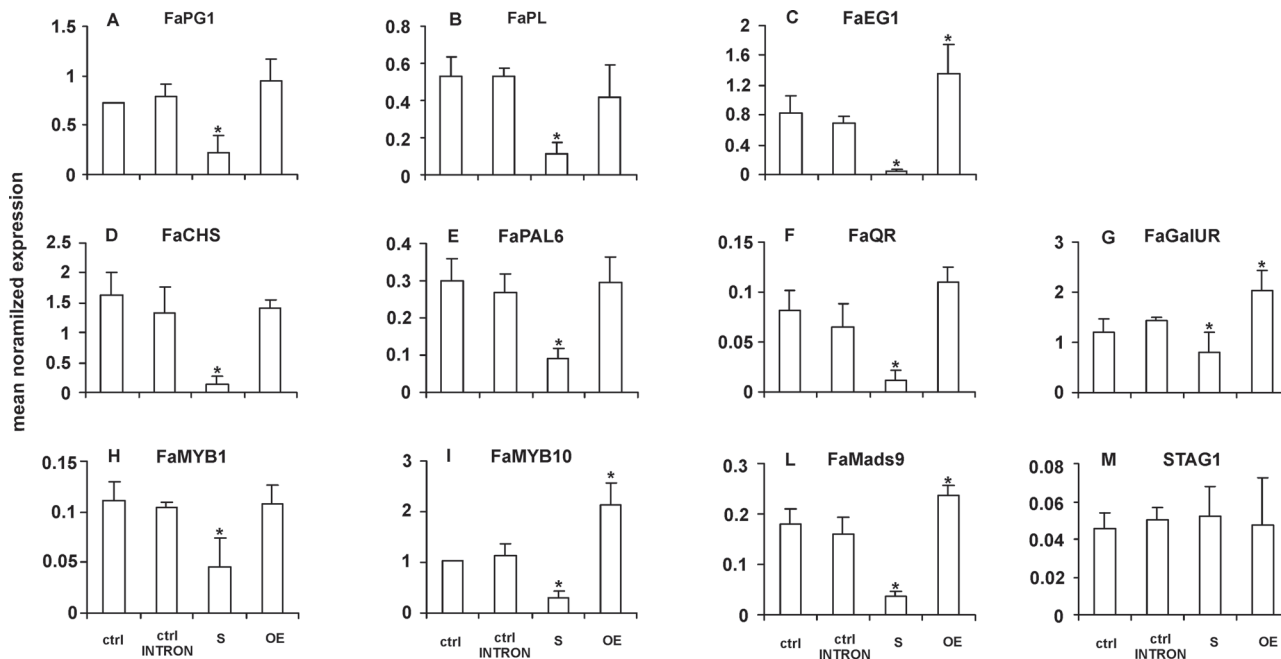


Fig. 2. Relative expression of ripening-related genes and transcription factor-encoding genes in *FaSHP*-silenced (S) and -over-expressing (OE) fruits. ctrl and ctrl INTRON values are respectively from fruits either wild-type or agro-injected with the control construct pBINplus_intron. Fruits agro-injected at the G2(2) stage were left attached to the plant until attainment of pink colour. Each value represents the mean of five independent fruits. *FaPG1*, Polygalacturonase; *FaPL*, pectate lyase; *FaEG1*, endo- β -1,4-glucanase; *FaCHS*, chalcone synthase; *FaPAL6*, phenylalanine ammonia lyase; *FaQR*, quinone oxidoreductase; *FaGalUR*, D-galacturonate reductase. Expression data (means of the normalized expression) were obtained by real-time PCR analyses. Bars are the standard deviations from the means. Asterisks indicate values significantly different by Student's *t* test from the control agro-injection ($P < 0.05$).

to be significantly reduced compared to controls, and the decrease seemed to be particularly strong in the case of the *FaEGI*, *FaCHS*, and *FaQR* genes (Fig. 2C, 2D, and 2F). Quite different was the result obtained with the *FaSHP*-over-expressing fruits where a significant increment (Fig. 2C and 2G) could be seen only for the *FaEGI* and *FaGalUR* genes.

We studied also the expression of a few transcription factor-encoding genes known to be involved in the development and ripening of strawberries. These genes encode two MYB transcription factors, FaMYB10 and FaMYB1, both of them suggested to be involved in the accumulation of fruit anthocyanins (Aharoni *et al.*, 2001; Lin-Wang *et al.*, 2010; Salvatierra *et al.*, 2013), and a MADS-box transcription factor, FaMADS9, belonging to the *SEPALLATA* group (Seymour *et al.*, 2011). All these genes showed an increasing expression during ripening, yet the *FaMYB10* gene was generally expressed at much higher levels than *FaMYB1*, and this difference was particularly relevant in fruits at the pink and red stages where the transcript amount of *FaMYB10* was about 10-fold higher than that of *FaMYB1* (Supplementary Fig. S3). Also for the transcription factor-encoding genes a significant decrease in their expression was observed in the case of *FaSHP* down-regulation, whereas in *FaSHP*-over-expressing fruits a significant parallel over-expression was observed for *FaMADS9* and *FaMYB10* but not for *FaMYB1* (Fig. 2L, 2H, and 2I).

Treatment of fruits with either auxin or ABA

The hormonal control of ripening is still poorly understood in the case of non-climacteric fruits. However, it has been shown that auxin can negatively control the ripening of strawberries since fruits treated with the hormone showed retarded coloration and repressed expression of a number of ripening-specific genes (Given *et al.*, 1988; Manning, 1994; Medina-Escobar *et al.*, 1997; Trainotti *et al.*, 1999; Aharoni and O'Connell, 2002). On the contrary, ABA has recently been shown to have a positive effect on ripening (Chen *et al.*, 2011; Jia *et al.*, 2011). Because of their apparently antagonistic effect on ripening, we decided to treat white fruits with either one or the other hormone to understand whether they can affect ripening by also affecting the expression of *FaSHP*. Treatment with auxin was effective even after 2 h in preventing the increase in *FaSHP* expression, while ABA started to be effective in increasing the expression of *FaSHP* at 4 h (Fig. 3A and 3B).

Fruits treated at either 6 or 18 h (see Supplementary Fig. S4) with each hormone were used to analyse the expression of the already studied marker genes. The antagonistic effect of the two hormones was evident for all of them in both the 6 and 18 h samples (Fig. 3C–3I). In the case of the *FaGalUR* gene a positive effect of ABA was always clear, while a negative effect of auxin on its expression was better seen in the 6 h sample. Interestingly, the two MYB genes and *FaMADS9* also appeared to be antagonistically regulated by the two hormones, with auxin having a negative effect and ABA a positive one on their expression (Fig. 3L, 3M, and 3N). On the contrary, no effect by any of the two hormones was observed in the case of *STAG1* (Fig. 3O).

Metabolomic analysis

Specific shifts in secondary metabolite accumulation have been shown to occur in strawberries during development and ripening of the receptacles and, to a lesser extent, of the achenes (Fait *et al.*, 2008). A semi-quantitative untargeted HPLC-ESI-MS analysis was carried out in strawberry fruits down- and up-regulated for *FaSHP* expression as well as in hormone-treated fruits, and the results are visualized as a heat map (Fig. 4). Seventy four peaks were putatively identified, including phenolic acids, anthocyanins, other flavonoids, ellagic acid derivatives, and triterpenoids. The details of metabolite putative identification are shown in Supplementary Table S1.

To study exactly the same material used for the analysis of gene expression, the metabolomics analyses were carried out on the same frozen and powdered fruits used for profiling the gene expression. The separation of the achenes from the receptacle was not made because the transient modification of gene expression involved only receptacles, hence the achenes were considered similar in all the examined samples.

The ripening of strawberries is marked by a colour shift from white to pink and finally to red, and this change of colour is due to anthocyanin accumulation. Both the *FaSHP*-down-regulated and the auxin-treated fruits showed lower levels of anthocyanins compared to the respective controls, while ABA-treated fruits accumulated higher amounts of pigments. On the contrary, the *FaSHP*-over-expressing fruits did not over-accumulate anthocyanins (Fig. 4). Besides anthocyanins, other metabolites either showed low levels in *FaSHP*-down-regulated and auxin-treated fruits or they over-accumulated after ABA treatment, including cinnamic and coumaric acid derivatives, one ferulic and one hydroxybenzoic acid derivative, and some flavonoids. Coumaric and ferulic acid derivatives have already been described as typical of red and pink receptacles, respectively (Fait *et al.*, 2008). Some metabolites, however, showed an opposite trend of accumulation, being over-accumulated in both *FaSHP*-down-regulated and NAA-treated fruits and showing lower levels after ABA treatment. They included caffeic acid derivatives and ellagitannins, already described as typical of white and pink receptacles and immature receptacles and achenes, respectively (Fait *et al.*, 2008).

Since the individual fruits showed variations in the level of the various secondary metabolites, the relationships between *FaSHP* expression and metabolite levels was investigated through OPLS in the various fruits, rather than by analysis of the absolute metabolite level. The OPLS analysis of control and *FaSHP*-down-regulated fruits resulted in a linear relationship between gene expression and the metabolite level (Supplementary Table S2). Within this analysis, the higher levels of *FaSHP* transcripts present in control fruits correlated positively with the accumulation of some coumaric, cinnamic, and benzoic acid derivatives and with anthocyanins (Supplementary Table S2 and Supplementary Fig. S5). The same metabolites were also strongly accumulated in ABA-treated samples and depleted in NAA-treated samples. On

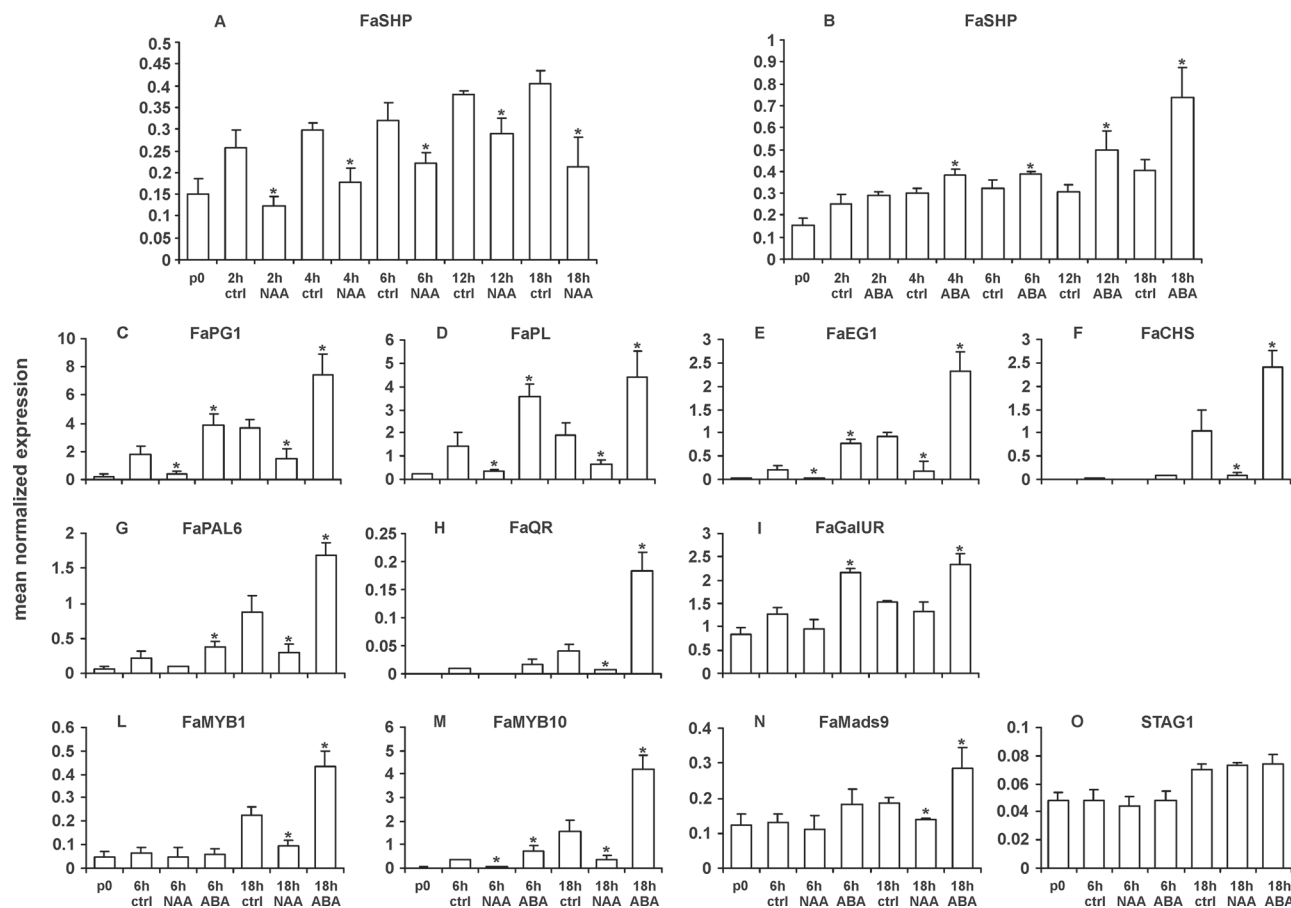


Fig. 3. Relative expression of genes following treatment of white fruits (W2) with either NAA or ABA. (A, B) Expression of the *FaSHP* gene in either NAA- (A) or ABA- (B) treated fruits. Treated and control fruits were sampled at 0, 2, 4, 6, 12, and 18h. Expression of ripening-related genes (C–I) and transcription factors (L–O) in 6 and 18h fruits either untreated (ctrl) or treated with one of the two hormones (NAA and ABA). Expression data (means of the normalized expression) were obtained by real-time PCR analyses. Bars are the standard deviations from the means. Asterisks indicate values significantly different by Student's *t* test from corresponding controls ($P < 0.05$).

the other hand, the low *FaSHP* transcript levels present in the *FaSHP*-down-regulated fruits correlated with higher levels of many metabolites, including various hydroxybenzoic and hydroxycinnamic acid derivatives, ellagic acid derivatives, and flavonoids. Interestingly, with the exception of only two metabolites, the same compounds were inhibited by ABA treatment, while their level in NAA-treated fruits was variable.

The OPLS analysis of control and *FaSHP*-up-regulated fruits did not result in any valid OPLS model, indicating the absence of any linear relationships between gene expression in up-regulated fruits and the metabolome. However, the heat map showed that *FaSHP*-up-regulated fruits accumulated higher levels of procyanidins. These metabolites, induced by NAA treatment and inhibited by ABA treatment, have been described to be typical of unripe receptacles (Fait *et al.*, 2008). Accordingly, their presence in the *FaSHP*-up-regulated fruits could be the consequence of a too-rapid increase in the *FaSHP* transcription factor that led to the appearance of the pink colour when the fruits were not yet “physiologically” pink.

Discussion

A C-type MADS-box gene (i.e. *STAG1*), belonging to the *euAGAMOUS* subgroup according to the definition by Kramer *et al.* (2004), was already known in strawberry (Aharoni *et al.*, 1999; Rosin *et al.*, 2003). In this work a second C-type gene belonging to the *PLENA* subgroup (a *SHATTERPROOF*-like one named by us *FaSHP*) was isolated and characterized. While *STAG1* was preferentially expressed during growth of the fruit, *FaSHP* showed ripening-specific expression and was generally expressed at much higher levels than *STAG1*. Modifications of the *FaSHP* gene expression were therefore performed to gain information about its role in the ripening of non-climacteric strawberries. The modifications consisted of either down-regulation or over-expression obtained by infiltrating strawberries still attached to the plant with a suspension of specifically modified *A. tumefaciens* cells.

Contrary to what had been found in tomato (Vrebalov *et al.*, 2009), in which the down-regulation of *TAGL1* (i.e. the orthologue of *FaSHP*) led to increased expression of *TAG1*

class of metabolite	OE	control	S	ABA	control	NAA	putative identification
anthocyanins	96	100	55	100	48	2	pelargonidin 3-O-glucoside
	76	100	16	100	29	3	pelargonidin-3-O-acetylglucoside
	100	30	27	100	31	6	cyandinin 3-O-glucoside
	100	93	67	100	94	80	cyandinin-3-O-malonylglucoside
	77	100	42	100	52	3	pelargonidin 3-O-malonylglucoside
cinnamic acid derivatives	77	100	69	100	59	3	cinnamoyl desoxyhexose d.
	33	100	12	100	75	5	cinnamoyl O-hexose
coumaric acid derivatives	83	100	73	100	96	79	coumaric acid hexose
	99	88	100	100	75	83	coumaroyl quinic acid
	100	84	88	64	100	98	coumaric acid hexose
caffeic acid derivative	86	72	100	100	69	42	caffeic acid hexose
	72	64	100	81	91	100	caffeic acid hexose
	100	65	100	61	84	100	caffeoyl quinic acid
	100	69	87	42	83	100	caffeic acid hexose d.
	79	63	100	51	80	100	caffeic acid d.
ferulic and dehydroferulic acid derivatives	81	83	100	104	98	100	ferulic acid hexose
	100	42	100	92	84	100	ferulic acid hexose d.
	100	100	89	38	100	68	dihydroferulic acid d.
	100	100	52	100	32	2	ferulic acid hexose d.
sinapic acid derivatives	100	99	94	100	98	89	sinapic acid hexose
	89	99	100	85	91	100	sinapic acid hexose
	35	44	100	83	93	100	sinapic acid hexose
	83	100	65	74	100	99	sinapic acid d.
hydroxybenzoic derivatives	65	79	100	70	100	90	ndihydroxybenzoic acid hexose
	100	76	88	62	100	99	dihydroxybenzoic acid hexose
	100	62	59	51	75	100	hydroxybenzoic acid hexose
	100	63	60	53	77	100	hydroxybenzoic acid hexose
	70	83	100	60	100	84	dihydroxybenzoic acid pentose
	87	58	100	70	100	63	hydroxybenzoyl hexose
	100	87	32	79	84	100	hydroxybenzoic acid d.
100	99	32	100	24	12	dihydroxybenzoic acid d.	
62	100	82	56	84	100	vanillic acid d.	
gallic acid derivatives	90	100	79	83	99	100	gallic acid pentose
procyanidin and flavan 3-ols	94	100	87	66	100	93	procyanidin trimer
	100	99	82	74	91	100	procyanidin dimer
	100	77	64	64	78	100	procyanidin trimer
	100	97	91	69	100	91	procyanidin trimer
	82	54	100	53	100	56	Procyanidin tetramer
	100	85	79	69	88	100	catechin
	100	86	84	54	82	100	procyanidin trimer
	92	100	82	59	95	100	Procyanidin tetramer
	100	91	86	67	100	99	procyanidin dimer
	100	87	66	100	90	100	Propelargonidin dimer
	83	91	100	54	100	99	procyanidin pentamer
	100	83	64	100	50	65	procyanidin trimer
	100	88	82	65	92	100	procyanidin dimer
	99	100	94	73	96	100	procyanidin tetramer
	100	91	85	58	90	100	procyanidin trimer
100	88	84	63	88	100	procyanidin dimer	
100	78	72	74	100	92	procyanidin trimer	
91	100	87	65	76	100	(Ep)afzelechin (or phloretin) hexose	
ellagic acid and ellagitannins	73	73	100	52	100	100	HHDP galloyl hexose
	83	91	100	55	86	100	ellagic acid hexose
	56	63	100	61	94	100	ellagic acid d.
	56	60	100	54	87	100	casuructin/potentillin like ellagitannin
	98	89	100	53	76	100	ellagic acid desoxyhexose
	92	100	97	60	73	100	ellagic acid pentose
	68	61	100	43	85	100	casuructin/potentillin like ellagitannin
	80	84	100	55	69	100	ellagic acid
100	75	86	53	80	100	ellagic acid d.	
flavonoids	57	54	100	72	100	92	trihydroxyflavone O-pentose
	96	100	94	66	90	100	kaempferol O-pentose
	92	95	100	84	100	91	trihydroxyflavone O-pentose
	94	100	96	94	94	100	trihydroxyflavone desoxyhexose
	93	100	53	100	38	4	tetrahydroxyflavanone O-hexose
	100	71	49	100	53	14	naringenin C-hexose
	83	100	78	100	50	78	quercetin -O-hexose
	31	100	90	79	81	100	quercetin O-glucuronide
	100	79	80	85	66	100	kaempferol O-hexose
	90	100	89	81	88	100	kaempferol-O-glucuronide
100	92	75	100	77	72	kaempferol-O-acetyl hexose	
100	25	91	31	38	100	kaempferol coumaroyl hexose	
triterpenoid derivatives	61	100	64	100	85	65	sesquiterpenoid hexose
	68	70	100	44	76	100	triterpenoid hexose
scale	1	10	20	1	10	20	
	30	40	50	30	40	50	
	60	70	80	60	70	80	
	90	100		90	100		

Fig. 4. Heat map of the 74 putatively identified metabolites in *FaSHP*-over-expressing (OE) and -silenced (S) fruits compared with their control fruits (fruits agro-infiltrated with the pBINplus_intron construct), and ABA- and NAA-treated fruits compared with their controls. The average relative content of each metabolite was expressed as a percentage with respect to the samples that showed the highest content of that metabolite (=100%). Since some anthocyanins were not easily detectable in negative ionization mode, the anthocyanin data come from the positive-ionization-mode data matrix; for all other metabolites the negative-ionization-mode data matrix was used. This figure is available in colour at JXB online.

(i.e. the orthologue of *STAG1*), neither the down-regulation nor the over-expression of *FaSHP* caused changes in the expression of *STAG1*. Therefore, under our experimental conditions the observed effects on ripening can be ascribed solely to the altered expression of *FaSHP*. Phenotypic macroscopic peculiarities were not observed but for variations in the number of days necessary to attain the pink colour starting from the moment of the agro-infiltration. This change was particularly significant in the case of the *FaSHP* down-regulation since about 9 days were necessary instead of the about 6 counted for the controls. Although smaller, a variation was also found in the case of the *FaSHP* over-expression which caused a slight acceleration of ripening with about 5 days taken to reach the pink stage. Thus, *FaSHP* appears to play a general positive role on the ripening of strawberries.

The significant delay in attaining the pink colour suggested a slackened expression of the genes involved in anthocyanin biosynthesis and a significantly down-regulated expression was actually observed for the *FaCHS* gene, which is involved in the synthesis of these coloured molecules in strawberry (Lunkenbein *et al.*, 2006). Also the two *MYB* genes, reported to be involved in the regulation of the pigment synthesis in strawberry fruits (Aharoni *et al.*, 2001; Lin-Wang *et al.*, 2010; Salvatierra *et al.*, 2013), were significantly down-regulated although this effect appeared particularly strong for *FaMYB10*.

A relevant characteristic of ripening is the softening of the fruit tissues. As evidenced in tomato (Brummell and Harpster, 2001; Carrari and Fernie, 2006) and peach (Trainotti *et al.*, 2003), many different genes are expressed at different moments throughout this process. In the *FaSHP*-down-regulated fruits the three softening-related genes appeared to be significantly repressed, especially *FaEG1*. Therefore, it seems that the process of softening may also be positively regulated by the *FaSHP* gene.

Two ripening-related genes involved in the production of secondary metabolites were also analysed. One of them (*FaQR*) is involved in the production of furofuran which is one of the main components of the strawberry aroma (Raab *et al.*, 2006), while the other gene (*FaGalUR*) is involved in the production of ascorbic acid (Cruz-Rus *et al.*, 2011). Both genes were significantly down-regulated, thus strengthening the idea that the *FaSHP* gene may have a wide regulatory function during strawberry ripening.

The down-regulation of *FaSHP* resulted in a metabolome typical of less-ripe fruits, characterized by lower levels of metabolites, such as coumaric acid derivatives and anthocyanins, that have been previously reported as typical of ripe receptacles, and higher levels of metabolites such as caffeic acid derivatives and ellagitannins, described as typical of unripe fruits (Fait *et al.*, 2008). Consistent with their characteristics as metabolites typical of ripe fruits, and with their relationship with *FaSHP* expression, coumaric acid derivatives and anthocyanins were also induced by ABA and repressed by NAA. Other metabolites, especially cinnamic acid derivatives, showed lower levels of accumulation in *FaSHP*-down-regulated fruits, and were induced by ABA and repressed by NAA. The latter metabolites behaved

like those previously described as typical of ripe fruits, and could therefore be considered as markers of the strawberry ripe fruit. On the other hand, the metabolites previously described as typical of unripe fruit were repressed by ABA but showed different responses to NAA. Thus, a complex relationship emerged between metabolites, *FaSHP*, ABA, and NAA. The metabolites whose accumulation was induced by the physiological expression of *FaSHP* in control fruits were also induced by ABA and inhibited by NAA. On the other hand, those metabolites whose accumulation increased in *FaSHP*-down-regulated fruits were inhibited by ABA but not necessarily by NAA.

The results of the *FaSHP* over-expression experiments did not appear as straightforward as in the case of the gene down-regulation; nonetheless, they were equally interesting. Only in the case of *FaEG1* and *FaGalUR* did the over-expression of *FaSHP* induce a significant increase in their expression, while all the other genes appeared unaffected. Since the acceleration of ripening was by 1 day only, it is probable that the ripening genes did not have enough time for a significant increase. Moreover, this finding suggests that the control of ripening by *FaSHP* is not wholly direct and might be mediated by other transcription factors.

The effect of the altered *FaSHP* expression on that of the two genes (*FaCHS* and *FaMYB10*) more directly involved in anthocyanin formation supports this idea. *FaMYB10* encodes a transcription factor that, when over-expressed in strawberry, caused an over-accumulation of anthocyanins (Lin-Wang *et al.*, 2010), and seems therefore to regulate more directly the downstream gene(s) coding for the enzyme(s) involved in the actual synthesis of the coloured molecules. In our case the *FaMYB10* gene was either down-regulated or over-expressed in a significant manner in parallel with the variations in *FaSHP* expression. On the contrary, the downstream gene *FaCHS* (Lunkenbein *et al.*, 2006) showed significantly altered expression only in the *FaSHP*-down-regulated strawberries that took 3 days more than controls to reach the pink stage. The results of the metabolomic analyses support this idea since significant variations in anthocyanin contents were only found in the *FaSHP*-down-regulated fruits.

Recently a negative role in anthocyanin formation has been shown for the *MYB1* strawberry gene. In fact, the gene down-regulation in white Chilean strawberries (*Fragaria chiloensis* ssp. *chiloensis*) resulted in a coloration of the fruits (Salvatierra *et al.*, 2013). Our data are in agreement with this idea since during normal ripening the *FaMYB1* gene is expressed at much lower levels than *FaMYB10* and, contrary to *MYB10*, in *FaSHP*-over-expressing fruits the *MYB1* transcript amount did not show any variation compared to controls.

Recently it was found in tomato that the RIN MADS-box transcription factor could bind to the promoter of genes coding for cell wall hydrolases, besides to the promoter of the two 1-aminocyclopropane-1-carboxylate synthase genes *ACS2* and *ACS4* (Fujisawa *et al.*, 2013). An informatic analysis carried out on the sequence of the *FaEG1* promoter (Spolaore *et al.*, 2003) evidenced the presence of CArG-box elements, the latter regarded as binding sites for MADS-box

transcription factors. Moreover, using databases of known position-specific scoring matrices (detailed in Materials and methods) CArG-box elements could be evidenced also in the promoter sequence of the *FaGalUR* gene (not shown). Accordingly, the regulatory activity performed by *FaSHP* on the *FaEGI* and *FaGalUR* genes might have been direct. Of course, we are aware that the presence of putative CArG-box elements does not demonstrate that the *FaSHP* protein actually binds to them.

Also in the case of the *FaMADS9* gene (of the *SEPALLATA* group) its expression changed significantly in parallel with either the down-regulation or the over-expression of *FaSHP*. This result suggests that the *FaMADS9* gene might be situated downstream of *FaSHP* along the regulatory network controlling ripening in strawberry.

It has long been known that for ripening to start the auxin content of the strawberry fleshy receptacle must decrease (Given *et al.*, 1988). Ripening-related processes that are sensitive to auxin comprise softening, change of colour, and the formation of aroma (Aharoni and O'Connell, 2002). Concomitant with the start of ripening, ABA is synthesized and contributes to the control of the process (Chen *et al.*, 2011; Jia *et al.*, 2011). Therefore, the two hormones are not significantly overlapping in their control of ripening, which is negative for auxin and positive for ABA.

The study of the *FaSHP* expression kinetics following treatments with either auxin or ABA has evidenced that both hormones can impact the expression of this important gene, although in a manner that reflects their role in ripening: the gene is down-regulated by auxin and up-regulated by ABA. A fragment of the *FaSHP* promoter (1612 bp upstream of the ATG codon) was obtained by RT-PCR and an informatic survey was carried out on its sequence. Interestingly, the promoter was found to harbour five putative domains for response to auxin and one putative domain for response to ABA (Supplementary Fig. S6), and this might explain why *FaSHP* can be regulated by both hormones.

The auxin effect on the expression of *FaSHP* was already clear after 2 h of treatment, while for ABA a clear effect was visible in the 4 h-treated sample. The ripening-related genes responded in the expected manner to the hormones but for *FaGalUR* which appeared positively regulated by ABA and only weakly repressed by auxin under our experimental conditions. Also three of the transcription factor-encoding genes (i.e. *FaMYB1*, *FaMYB10*, and *FaMADS9*) responded in the expected manner to the hormones although not in the same measure. On the contrary, the other C-type MADS-box gene (*STAG1*) appeared to be insensitive to both hormones, thus suggesting once more its lack of involvement in the ripening of strawberries.

In the climacteric tomato and peach fruits the initial growth stage is characterized by high auxin content while during ripening ethylene plays a fundamental role (Gillaspy *et al.* 1993; Miller *et al.*, 1987). Recently, ABA has also been shown to participate in the control of ripening together with ethylene (Sun *et al.*, 2010). During the passage from auxin to ethylene control the tomato gene *TAGL1* increases

its expression and has a role in ripening (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009). In non-climacteric strawberries there is an early growth stage under the control of auxin followed by ripening that seems to be under ABA control (Perkins-Veazie 1995; Chen *et al.*, 2011; Jia *et al.*, 2011). In this fruit, during the passage from auxin to ABA control, the *FaSHP* gene (orthologous of *TAGL1*) increases its expression and begins to exert its important regulatory role on the ripening process. Thus the characteristic changes of the secondary metabolome that accompany ripening (i.e. accumulation of metabolites typical of ripe fruit and depletion of those typical of unripe fruit) appear to be under *FaSHP*, ABA, and NAA control, where ABA and *FaSHP* act as inducers of ripe-typical metabolite accumulation and NAA as repressor of ripe-typical metabolites. On the other hand, the unripe-typical metabolome depends on the low level of expression of *FaSHP* and the low level of ABA, while only some metabolites typical of unripe fruit seem to be under NAA control.

TAGL1 and *FaSHP* belong to the *PLENA* subclade of the C-type MADS-box genes, and both of them appear to increase their expression and to positively impact fruit ripening when the initial control on fruit growth exerted by auxin is handed over to either ethylene or ABA. However, *TAGL1* is active in tomato which is a botanical climacteric fruit while *FaSHP* is active in strawberry which is a non-climacteric receptacle-derived fruit. This suggests that the MADS-box genes of the *PLENA* subgroup may impact the ripening of fleshy structures with fruit function independently of their anatomical origin.

Supplementary material

Supplementary material is available at *JXB* online.

[Supplementary File 1.](#) Supplementary materials and methods.

[Supplementary Fig. S1.](#) Pictures of *FaSHP*-down-regulated, *FaSHP*-up-regulated, and control fruits.

[Supplementary Fig. S2.](#) Relative expression profiles of ripening-related genes.

[Supplementary Fig. S3.](#) Relative expression profiles of ripening-related transcription factor-encoding genes.

[Supplementary Fig. S4.](#) Pictures of NAA- and ABA-treated fruits.

[Supplementary Fig. S5.](#) OPLS analysis of *FaSHP*-down-regulated and control fruits.

[Supplementary Fig. S6.](#) Bioinformatic analysis of the *FaSHP* gene promoter.

[Supplementary Table S1.](#) Metabolite putative identification.

[Supplementary Table S2.](#) Pq(corr) loadings of OPLS analysis and comparison with metabolite levels in agro-injected and hormone-treated fruits.

[Supplementary Table S3.](#) Sequences of the oligonucleotides used in the real-time PCR experiments.

[Supplementary Table S4.](#) GenBank accession numbers of the sequences used for the construction of the phylogenetic tree.

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