Characterizing the involvement of FaMADS9 in the regulation of strawberry fruit receptacle development

José G. Vallarino1,2,†, Catharina Merchante1,†, José F. Sánchez-Sevilla2,3, María AngelinesLuis Balaguer4,a, Delphine M. Pott1,2, Maria T. Ariza1,2, Ana Casañal1, David Posé1,2, Amalia Vioque1, Iraida Amaya2,3, Lothar Willmitzer2, Roberto Solano6,b, Rosangela Sozzani4,7, Alisdair R. Fernie5, Miguel A. Botella1,2, James J. Giovannoni8, Victoriano Valpuesta1,2,* and Sonia Osorio1,2,* (Received 5 November 2018; revised 3 September 2019; accepted 8 September 2019.

† Correspondence (Tel +34 952131932; email valpuesta@uma.es (V.V.) and Tel +34 952134271; email sosorio@uma.es (S.O.))

Present address: Precision Biosciences, Inc., Durham, NC, USA

*These authors contributed equally to the article.

Abstract

FaMADS9 is the strawberry (Fragaria x ananassa) gene that exhibits the highest homology to the tomato (Solanum lycopersicum) RIN gene. Transgenic lines were obtained in which FaMADS9 was silenced. The fruits of these lines did not show differences in basic parameters, such as fruit firmness or colour, but exhibited lower Brix values in three of the four independent lines. The gene ontology MapMan category that was most enriched among the differentially expressed genes in the receptacles at the white stage corresponded to the regulation of transcription, including a high percentage of transcription factors and regulatory proteins associated with auxin action. In contrast, the most enriched categories at the red stage were transport, lipid metabolism and cell wall. Metabolomic analysis of the receptacles of the transformed fruits identified significant changes in the content of maltose, galactonic acid-1,4-lactone, proanthocyanidins and flavonols at the green/white stage, while isomaltose, anthocyanins and cuticular wax metabolism were the most affected at the red stage. Among the regulatory genes that were differentially expressed in the transgenic receptacles were several genes previously linked to flavonoid metabolism, such as MYB10, DIV, ZFN1, ZFN2, GT2, and GT5, or associated with the action of hormones, such as abscisic acid, SHP, ASR, GTE7 and SnRK2.7. The inference of a gene regulatory network, based on a dynamic Bayesian approach, among the genes differentially expressed in the transgenic receptacles at the white and red stages, identified the genes KAN1, DIV, ZFN2 and GTE7 as putative targets of FaMADS9. A MADS9-specific CArG box was identified in the promoters of these genes.

Keywords: strawberry, fruit ripening, quality.

Introduction

Strawberry is a non-climacteric fruit, and several studies have analysed the metabolic (Fait et al., 2008) and cell wall changes (Jiménez-Bermúdez et al., 2002; Quesada et al., 2009) that occur during fruit ripening, as well as changes in gene expression associated with this process (Aharoni and O’Connell, 2002; Härtl et al., 2017; Pillet et al., 2015; Sánchez-Sevilla et al., 2017). Recently, a number of reports have shed light on two key elements of the strawberry fruit developmental programme of strawberry fruit: the involvement of hormones and the participation of specific transcription factors.

Early studies reported that auxin plays an essential role in the regulation of strawberry fruit growth and ripening because the growth of the green receptacles is dependent on auxin delivery from the achenes (Mezzetti et al., 2004; Nitsch, 1950). However, a recent analysis of the transcriptome in developing fruits showed significant expression of several auxin biosynthetic and signalling genes in the ripening receptacles (Estrada-Johnson et al., 2017). In addition to auxin, other hormones have been reported to be involved in fruit development, such as gibberellins (Csukasi et al., 2011), abscisic acid (Chai et al., 2011; Jia et al., 2011), jasmonates (Concha et al., 2013; Mikkun and Singh, 2009) and brassinosteroids (Chai et al., 2013). In contrast to the situation in climacteric fruits, ethylene does not play a key role in the onset of the ripening process (Perkins-Veazie, 1995). However, Merchante et al. (2013) described a role for this hormone in some specific ripening-associated molecular changes, despite the lack of a general effect on fruit ripening.

Studies regarding the regulatory genes of the strawberry fruit ripening process are limited, and most of the studies have examined the roles of these genes in the metabolic changes that occur with ripening. In this context, two transcription factors (TFs), namely FaMYB1 and FaMYB10, have been reported to play a major role in the regulation of...
phenylpropanoid, flavonoid and anthocyanin biosynthesis during ripening (Aharoni et al., 2001; Lin-Wang et al., 2010; Medina-Puche et al., 2014). Additionally, studies on the three components of the complex that regulates flavonoid biosynthesis identified four TFs, namely FaMYB9/FaMYB11, FabHLL3 and FaTTG1, as positive regulators of the biosynthesis of proanthocyanidins (Schaart et al., 2013). Correlation analysis of the transcriptomes of ripening fruits also identified three TFs of the MYB, TCP and SCL families that were positively correlated with flavonoid accumulation in ripening fruits (Pillet et al., 2015). Recently, other TFs have been reported to regulate the gene expression of eugenol synthase2 (Medina-Puche et al., 2015; Molina-Hidalgo et al., 2017), which is involved in the synthesis of eugenol (Áragüez et al., 2013).

To date, few TFs have been identified as being linked to the role of hormones in strawberry fruit ripening. That said, we previously identified a GAMYB TF gene, FaGAMYB, as a key regulatory player in the initiation of strawberry receptacle ripening that acts upstream of ABA and sucrose signalling (Vallarino et al., 2015). It has also been proposed that the C-type MADS-box gene SHATTERPROOF-like (FaSHP) might play a modulatory role in strawberry fruit ripening acting, either directly or indirectly, via other TFs during the transition from auxin-to ABA-mediated control (Daminato et al., 2013).

In climacteric fruit such as tomato, the RIN transcription factor has been linked to ripening-associated enhancement of ethylene levels (Giovannoni, 2007; Vrebavlov et al., 2002). Recently, by examination of RIN knockout mutants, RIN was shown to be required for complete normal ripening of tomato fruits (Ito et al., 2017). Similarly, homologous MADS-box genes in other species have been reported to play a major role in the development and ripening of other climacteric fruits, such as peach (Tadiello et al., 2009), banana (Elitzur et al., 2010) and oil palm (Tranbarger et al., 2011). In non-climacteric fruits such as bilberry (Jaakola et al., 2010), the putative orthologue of the tomato RIN has been demonstrated to be involved in fruit ripening. In strawberry, silencing of the FaMADS9 gene, an orthologue of the tomato RIN, altered the normal development of the achene and receptacle, and transcriptomic analysis of the silenced fruits revealed pleiotropic effects on gene expression (Seymour et al., 2011). By analysis of the strawberry fruit transcriptome, we found that FaMADS9 presented weak expression in the achenes and high expression in the receptacles, showing two main peaks, one at the green developmental stage and the other at the red developmental stage (Sánchez-Sevilla et al., 2017). In this work, we complement the preliminary study of this gene (Seymour et al., 2011) via the generation of four independent strawberry transgenic lines that were silenced in the FaMADS9 gene and present an in-depth analysis. Our data support a major role of the FaMADS9 gene in not only the orchestration of the metabolic changes that occur during fruit ripening, but also the possible involvement of auxin and abscisic acid in the fruit developmental programme, and, in particular, in the formation and synthesis of the fruit cuticle.

Results
Silencing of FaMADS9 does not produce a visually apparent phenotype in strawberry fruits
To dissect the role played by FaMADS9 in the ripening receptacles, we performed stable RNAi-mediated FaMADS9 gene silencing in plants of the Camarosa cultivar. Four transgenic lines (L6, L7, L23 and L27) were selected based on the significant reduction in FaMADS9 expression in these lines. Although phenotypic differences between control and FaMADS9-silenced lines were reported when the cultivar Calypso was used (Seymour et al., 2011), the transformed fruits herein showed a normal appearance (Figure 1a). Analysis of other fruit characteristics, such as firmness and total soluble solid content (Brix), showed that three of the silenced lines (L7, L23 and L27) exhibited a significantly reduced Brix content, whereas no significant changes were observed in fruit firmness (Figure 1b,c). Given that FaMADS9 is expressed in the early stage of achene development (Sánchez-Sevilla et al., 2017), as well as in flowers (Seymour et al., 2011), we next evaluated the effect of FaMADS9 silencing on fruit abortion and fruit yield. The silenced lines exhibited no significant changes in these traits (Figure 1d,e).

Evaluation of the changes in the receptacle transcriptome of silenced lines identifies regulatory genes associated with hormone activity
The two silenced lines (L6 and L7) that showed strong reduction in FaMADS9 expression and the control were selected for RNA sequencing (RNAseq) expression analysis of the receptacles at two different stages, white and red (Figure 1f,g; Table S1). Because the reads were mapped on the Fragaria vesca genome (Edger et al., 2018), the differentially expressed genes were named hereafter based on the orthologue in F. vesca. At the white stage, a total of 2670 genes were significantly differentially expressed, while at the red stage, 1943 genes were differentially expressed (Table S2). Notably, only one of the 34 MADS genes expressed in fruits (Sánchez-Sevilla et al., 2017) was down-regulated in the silenced receptacles, namely FaMADS9 (FvH4_6g64220), the expression of which decreased more than 90% in both the white and red stages (Figure 1f). This result was confirmed when the expression was evaluated by qRT-PCR (Figure 1g). However, another FaMADS gene, namely SHATTERPROOF1-like (SHP1; FvH4_6g37880), was up-regulated in both the white and red receptacles (Table S3; Figure S1). The differential expression of these two MADS genes was also confirmed by qRT-PCR, as was the expression of three other MADS genes (FvH4_3g06720, FvH4_5g13510 and FvH4_5g35410) that were not differentially expressed in the RNAseq study (Table S3, Figure S1).

Analysis of the enriched functional MapMan categories (Thimm et al., 2004) of the differentially expressed genes was performed at the two developmental stages (Table S4). In the white receptacle, the most enriched category corresponded to RNA (Bincode ‘27.3.’; 26%) (Figure 2a), which included many TFs (over 82%). Among the TF families, the sub-category of auxin response factors (ARFs) (Bincode ‘27.3.4.’) was also enriched. This category included nine members of the ARF family from the seventeen that are expressed in strawberry fruits (Estrada-Johnson et al., 2017), all but one (ARF16c) of which were down-regulated, mostly at the white stage (Table S5). Additionally, there was a change in the expression of seven members of the regulatory AUX/IAA genes, all of which were down-regulated by IAA11 (Table S5). The only two genes associated with auxin metabolism that were differentially expressed in the transgenic receptacles were GH3.6 (FvH4_2g24250), which was up-regulated in the white receptacles, and GH3.17 (FvH4_4g22430), which was down-regulated in red receptacles (Table S5). The next most enriched sub-category was the DOF family of TFs (Bincode ‘27.3.8.’), which included FaDOF1 (FvH4_5g05330) and FaDOF2 (FvH4_2g14390),
the latter being involved in eugenol production in the ripe receptacles (Molina-Hidalgo et al., 2017). While FaDOF1 was down-regulated in the white receptacles of the transgenic lines, FaDOF2 was up-regulated (Table S2).

**Cell growth processes and energetic metabolism were altered in the silenced lines**

The next most enriched MapMan category in the white receptacles was miscellaneous (Bincode ‘26’, 20%) (Figure 2a), which includes a diverse group of genes, prominent among which are the UDP-glucosyl/glucuronosyl transferases (32 genes) (Table S4). The enrichment of this MapMan category, along with other highly represented categories such as cell (Bincode ‘31’; 12%), cell wall (Bincode ‘10’; 10%) and transport (Bincode ‘34’; 15%) (Figure 2a; Table S4), suggests a considerable role of FaMADS9 in the growth processes associated with early-stage receptacle development. Thus, the third most enriched category, transport, included 12 genes corresponding to major intrinsic proteins (NIP,
stages. (Figure S2b).

Lines L6 and L7 grouped together at the green and red stages the samples on the basis of developmental stage and genotype. Therefore, primary metabolites were analysed in the receptacles (Figure 2a) and lipids in the red receptacles (Figure 2b).

Various metabolic categories were enriched in the differentially expressed genes: carbohydrates and amino acids in the white receptacles (Figure 2a) and lipids in the red receptacles (Figure 2b). Therefore, primary metabolites were analysed in the receptacles of the transgenic lines (Table S6). One annotated as NIP1-1-like aquaporin (FvH4_6g08000) was up-regulated in the white receptacles.

Metabolic changes in the receptacles of FaMADS9-silenced lines lead to alterations in starch and ascorbate metabolism

Various metabolic categories were enriched in the differentially expressed genes: carbohydrates and amino acids in the white receptacles (Figure 2a) and lipids in the red receptacles (Figure 2b). Therefore, primary metabolites were analysed in the receptacles of the four silenced lines (L6, L7, L23 and L27) at the green, white and red stages. Both principal component analysis (Figure S2a) and hierarchical clustering analysis (Figure S2b) clearly grouped the samples on the basis of developmental stage and genotype. Lines L6 and L7 grouped together at the green and red stages (Figure S2b).

However, the exact pattern of clustering varied among the three stages. A heat map exhibiting the values obtained for all the samples is presented in Figure 3 (Table S7). Metabolic changes were restricted to a few metabolites and only to green and red receptacles. Two of these metabolites are maltose and isomaltose, which are disaccharides produced by the hydrolysis of starch by amylase and isoamylase, respectively. While maltose levels decreased in the green receptacles of the transgenic lines compared to the control, isomaltose levels increased in the red receptacles. These results are consistent with the expression of the strawberry amylase and isoamylase genes in ripening receptacles and their altered expression of these genes in FaMADS9-silenced lines. In the white receptacles, the expression of three β-amylases (FvH4_4g17110, FvH4_5g20800 and FvH4_4g05230) was significantly decreased in transgenic receptacles, whereas the expression of one isoamylase (FvH4_5g36440) was enhanced (Table S8). At the red stage, no significant decrease was observed in the isoamy- lase levels, while one β-amylase (FvH4_4g05230) was down-regulated and another β-amylase (FvH4_3g29220), with very low expression levels, was up-regulated (Table S8).

Another change observed in all the transgenic lines corresponded to galactonic acid-1,4-lactone, the level of which decreased in the receptacles of the transgenic lines, at the white and red stages, compared to the control (Figure 3). Two pathways have been proposed to be responsible for L-ascorbic acid biosynthesis in strawberry, with galactonic acid-1,4-lactone being the final intermediate (Valpuesta and Botella, 2004). Our RNAseq analysis of the silenced receptacles revealed that at the white stage, two genes encoding enzymes of the L-galactose pathway were down-regulated (Table S9). In addition, metabo-lomic profiling of the green receptacles showed a decrease in ascorbate content in the transgenic lines compared to the control (1.0 ± 0.2; 0.7 ± 0.1; 0.5 ± 0.2; 0.8 ± 0.1; 0.6 ± 0.1 for WT, L6, L7, L23 and L27, respectively; data are normalized to the mean response calculated for the WT). Thus, a diminished flux through the L-ascorbic acid biosynthetic pathway was expected in the transgenic white and red receptacles, which would explain the low galactonic acid-1,4-lactone content at these stages.

The transcriptome of the red receptacles of FaMADS9-silenced lines indicates specific changes in the cell wall

Functional analysis of differentially expressed genes in the red receptacles (Table S2) was performed according to the MapMan categories (Thimm et al., 2004). Enriched categories were restricted to four (Figure 2b, Table S4). The most enriched category at this time point was transport (37%). Genes included in this category represent a wide number of gene families associated with very diverse transport systems, with the major intrinsic protein category being highly represented. Five of the twelve genes in this category were down-regulated (Table S6). The next most enriched category was cell wall (Bincode ‘10’; 26%) (Figure 2b), including an enriched sub-category of cell wall precursor synthesis (Bincode ‘10.1’) (Table S4). Most of the genes down-regulated in this category correspond to the synthesis and modification of cell wall components, such as different classes of proteins and glycopolymers (Table S1).

Since we did not observe differences in the firmness of transgenic fruits (Figure 1c), the expression of genes reported to be involved in cell wall disassembly and degradation during strawberry fruit ripening (Benítez-Burraco et al., 2003; Jiménez-Bermúdez et al., 2002; Molina-Hidalgo et al., 2013; Quesada et al., 2009) was analysed (Table S10). Only polygalacturonases were up-regulated in the transgenic fruits, with both PG1 (FvH4_6g041380) and PG2 (FvH4_7g15040) up-regulated in the white receptacles, and only PG1 up-regulated in the red receptacles.

Lipid metabolism of epidermal cells of the red receptacles is altered in silenced lines

Lipid metabolism (Bincode ‘11’) is another enriched category (20%) of differentially expressed genes in the ripe receptacle (Figure 2b). In this category, all the genes corresponding to fatty
acid synthesis and elongation were down-regulated, whereas most of the lipases were up-regulated (Tables S1 and S4). In relation to the genes responsible for wax metabolism, several of the putative orthologues of the Arabidopsis genes (Bernard and Joubès, 2013) were down-regulated, at the white and red stages, in particular CER26 (FvH4_4g13360), CERS/CER7/ABCG12 (FvH4_3g28460), CER6 (FvH4_4g27420), KCR1 (FvH4_4g25050), LACS2 (FvH4_2g07450), HDG1 (FvH4_7g13350) and β-amyrin 28-oxidase (FvH4_3g11590) (Table S11). Moreover, the putative orthologue of the HDG1 gene (FvH4_7g13350) in strawberry, a transcriptional regulator of cuticular wax biosynthesis in Arabidopsis (Lee and Suh, 2015), was down-regulated in the white and red receptacles of the silenced lines (Table S11). Other regulatory genes of wax biosynthesis and cuticle formation, such as WIN1/SHINE1 (FvH4_6g29930) and SHINE3 (FvH4_2g29150) (Aharoni et al., 2004; Shi et al., 2013), were also down-regulated in the white receptacles (Table S11). Therefore, we next measured the cuticular wax in ripe fruits of the four FaMADS9-silenced lines and the control. There was a significant increase in the content of the highly abundant n-alkanes, such as C27- and C29-alkanes, in the four lines, and of C23-alkanes in three of the four lines (Table 1). In addition, the α- and β-amyrin triterpenoid content of the cuticular wax was also significantly enhanced in the four transgenic lines (Table 1). The annotated genes associated with amyrin metabolism that were altered in the transgenic lines were β-amyrin 28-oxidase (FvH4_3g11590), which was up-regulated in the white and red receptacles of these lines in comparison with the control and a predicted β-amyrin synthase (FvH4_6g36520), which was also up-regulated, but only in the white receptacles (Table S11); the latter is the first enzyme proposed to modify β-amyrin into oleanolic acid (Han et al., 2013).

Waxes cover or are embedded into the cutin polymer, forming the plant cuticle (Domínguez et al., 2015). Since we found

Figure 3 Heat map of primary metabolism in the receptacle of FaMADS9-silenced lines at three ripening stages. A colour-coded matrix represents the mean values of the metabolite intensity which has been log2-transformed and mean-centred.
changes in the epicuticular wax, we focused on the other component of the cuticle, the cutin. For this purpose, we evaluated the expression of the strawberry orthologue (FvH4_6g01380) of the cutin synthases of tomato, CD1 (Yeats et al., 2012a); the orthologue was down-regulated in the transgenic lines at the white stage (Table S11). The differential expression of this gene along with two selected wax biosynthesis genes, namely CER2 and CER26, was checked by qPCR in the white and red receptacles (Figure S3a). Silencing of CER26 and CD1 was confirmed, while silencing of CER2 was significant only at the white stage. Analysis of the expression of these genes in different fruit parts shows that CER26 and CD1 are mostly expressed in the epidermal cell-enriched surface of the fruit (Figure S3b).

In tomato fruits, it has been reported that cuticular wax composition is changed in ABA-deficient mutants (Martin et al., 2017). Previous studies have shown that ABA plays an important role in the regulation of strawberry fruit ripening (Chai et al., 2011; Jia et al., 2011; Li et al., 2015). Here, we found that three key genes involved in the synthesis of ABA, namely NCED1 (FvH4_3g16740), NCED2 (FvH4_3g05440) and NCED3 (FvH4_3g16730), the expression of which increases during receptacle ripening (Sánchez-Sevilla et al., 2017), were significantly down-regulated (over 70%) in the red receptacles of FaMADS9-silenced lines (Table S2). Then, the ABA level was measured in the receptacles of the transgenic fruits and the control. The results showed a reduction in all the lines between 22% and 49% (Table S12).

### Secondary metabolism in the red receptacle of silenced lines shows clear changes in the phenylpropanoid pathway

Although there was not an apparent colour change phenotype in the fruits of the transgenic lines (Figure 1a), due to the elevated number of DE genes in transgenic receptacles (Table S2), changes in other colourless secondary metabolites were expected. Thus, analysis of secondary metabolism was performed in the receptacle of four independent transgenic lines at the green, white and red stages (Figure 4; Table S13). At the green stage, there was an increase in procyanidins, propelargonidins and flavan-3-ols levels in the transgenic lines. In contrast, the levels of galloyl derivatives, quercetin and kaempferol derivatives, terpenoid derivatives, eriodicytol hexoses, and ellagitannins were diminished in the green receptacles of the transgenic lines. In the case of ellagitannins, two glycosyltransferases (GTs) that are involved in ellagitannin metabolism (FvH4_2g05060 and FvH4_2g05090) (Schulenburg et al., 2016) were up-regulated in the transgenic green receptacles (Table S2). Significant changes in the red receptacles were observed in the levels of naringenin chalcone hexose, which increased, and the anthocyanins (Figure 4; Table S13). Of the anthocyanins, while the cyanidin derivative content was low in the transgenic receptacles, the pelargonidin derivative content was high. Other compounds, such as ellagic acid, its derivatives, and hydroxycinnamic acid and benzoic acid derivatives, were invariant across the genotypes at all the developmental stages studied.

### Table 1 Wax constituents in FaMADS9-silenced fruits (relative per cent) identified in strawberry cuticles at the red stage.

| Wax constituents | WT    | L6    | L7    | L23   | L27   |
|------------------|-------|-------|-------|-------|-------|
| n-alkanes        |       |       |       |       |       |
| Docosane (C22)   | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.1 | 0.1 ± 0.0 | 0.1 ± 0.1 |
| Tricosane (C23)  | 2.1 ± 0.3 | 2.6 ± 0.4 | 4.1 ± 0.5 | 4.9 ± 0.3 | 5.8 ± 0.4 |
| Tetracosane (C24) | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 |
| Hexacosane (C26) | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 |
| Heptacosane (C27) | 3.7 ± 0.2 | 5.2 ± 0.3 | 6.9 ± 0.3 | 7.3 ± 0.6 | 6.5 ± 0.4 |
| Octacosane (C28) | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.0 | 0.3 ± 0.1 | 0.4 ± 0.1 |
| Nonacosane (C29) | 7.1 ± 0.7 | 13.8 ± 0.3 | 15.9 ± 0.5 | 14.4 ± 0.8 | 18.6 ± 0.9 |
| Triactonate (C30) | 0.8 ± 0.2 | 0.7 ± 0.2 | 1.0 ± 0.3 | 1.1 ± 0.3 | 0.9 ± 0.3 |
| Hentriactonate (C31) | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.2 | 0.1 ± 0.1 |
| Dotriactonate (C32) | 0.6 ± 0.1 | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.6 ± 0.3 | 0.7 ± 0.2 |
| iso-alkanes      |       |       |       |       |       |
| 2-methyl triactonol (iso-C31) | 2.4 ± 0.5 | 2.9 ± 0.7 | 2.2 ± 0.6 | 2.7 ± 0.6 | 2.2 ± 0.8 |
| 2-methylhentriactonane (iso-C32) | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.2 | 0.3 ± 0.1 |
| n-alkan-1-ols    |       |       |       |       |       |
| Docosanol (C22)  | 1.4 ± 0.5 | 1.1 ± 0.4 | 1.7 ± 0.5 | 1.1 ± 0.5 | 1.5 ± 0.3 |
| Tricosanol (C23) | 2.2 ± 0.5 | 2.0 ± 0.3 | 1.9 ± 0.4 | 2.2 ± 0.5 | 1.9 ± 0.5 |
| Tetracosanol (C24) | 0.2 ± 0.1 | 0.2 ± 0.0 | 0.2 ± 0.2 | 0.2 ± 0.1 | 0.1 ± 0.1 |
| Hexacosanol (C26) | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.2 ± 0.2 | 0.3 ± 0.1 |
| Octacosanol (C28) | 0.5 ± 0.1 | 0.5 ± 0.3 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.9 ± 0.2 |
| Nonacosan (C29)  | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.2 | 0.2 ± 0.1 |
| Triactonate (C30) | 0.3 ± 0.1 | 0.2 ± 0.2 | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 |
| Amyrins          |       |       |       |       |       |
| ß                | 4.7 ± 0.6 | 16.3 ± 0.5 | 11.6 ± 0.4 | 15.8 ± 0.5 | 18.3 ± 0.6 |
| ß                | 8.9 ± 0.5 | 9.4 ± 0.6 | 7.8 ± 0.6 | 8.6 ± 0.7 | 9.9 ± 0.7 |
| ß                | 17.7 ± 0.4 | 20.5 ± 0.5 | 22.8 ± 0.4 | 19.9 ± 0.2 | 24.3 ± 0.6 |

Data in bold indicate statistically significant differences from WT as determined by Student’s t-test (P < 0.05).
These changes in secondary metabolites along with changes in the expression of genes involved in their associated biosynthetic pathways are summarized in Figure 5. In addition to the diminished expression of the 4-coumarate-coenzyme ligase (4CL) genes in the white receptacles (Table S14), there was an increase in the expression of genes involved in the first steps of the flavonoid pathway (CHS1, FvH4_7g01160; CHI1, FvH4_7g20870; CHI3, FvH4_7g25890; F3H, FvH4_1g11810) (Table S1), which may explain the increase in proanthocyanidins levels in the green receptacles (Figures 4 and 5). The down-regulation of the FLS3 gene (FvH4_5g29430) in the silenced receptacles would explain the decreased levels of flavonols and their derivatives that were observed in the green receptacles (Figures 4 and 5). At the red stage, the only significant changes in gene expression corresponded to the early genes of the phenylpropanoid pathway, such as PAL1 (FvH4_6g16060), PAL2 (FvH4_7g19130), CAH (FvH4_3g40570), 4CL (Table S14), and F3H (FvH4_1g11810) which were down-regulated (Figure 5). This diminution was enhanced for the PAL genes and should affect all of the downstream metabolites in the pathway. The down-regulation of F3'H (FvH4_5g14010) at the white stage (Figure 5; Table S1) would explain the diminished content of cyanidin hexose in the red receptacles of transgenic fruits (Figure 4; Table S13).

The analysis of MYB10 (FvH4_1g22020), a gene involved in the regulation of the flavonoid pathway in strawberry fruits (Medina-Puche et al., 2014), showed that the expression of this gene was significantly up-regulated in the white receptacles (Table S2). Other TFs for which expression was correlated with flavonoid metabolism, such as TCP1 (FvH4_6g16170), ZFN2 (FvH4_7g01060) and DIV (FvH4_6g11950) (Pillet et al., 2015), were down-regulated in transgenic red receptacles (Table S2). In contrast, other genes associated with anthocyanin production in strawberry, such as the four members of the RAP family (FvH4_1g27460, FvH4_6g38760, FvH4_2g25200, FvH4_2g25210), encoding GST anthocyanin transporters (Luo et al., 2018), were up-regulated in white receptacles of the silenced lines.

Analysis of the gene regulatory network in the ripening receptacles identified transcription factors associated with FaMADS9

The high number of differentially expressed genes found in the FaMADS9-silenced fruits reveals the key role played by the FaMADS9 gene in the fruit ripening process and is a consequence of both direct and indirect transcriptional effects of the gene silencing. To gain insight into the possible relationships among these differentially expressed genes, two gene expression data sets were used to infer a gene regulatory network (GRN). GRN inference was performed using GENIST, based on a dynamic Bayesian network (DBN) inference algorithm, which has exceptional utility when starting from time course or developmental

**Figure 4** Heat map of identified secondary metabolites in FaMADS9-silenced lines and WT strawberry at different developmental stages. A colour-coded matrix represents the mean values of the metabolite intensity which has been log10-transformed and mean-centred.
Figure 5  Pathway analysis of genes and enzymes involved in shikimate, phenylpropanoid and flavonoid–anthocyanin pathways. The heat maps represent the transcript (garnet-green) and metabolites (red-blue) data log2-scaled and mean-centred. FaANS, anthocyanidin synthase; FaANR, anthocyanidin reductase; FaCA4H, cinnamic acid 4-hydroxylase; FaCHI, chalcone isomerase; FaCHS, chalcone synthase; Fa4CL, 4-coumaroyl-CoA ligase; FaDFR, dihydroflavonol reductase; FaFGT, flavonoid glucosyltransferase; FaF3H, flavanone 3-hydroxylase; FaF3’H, flavonoid 3’-hydroxylase; FaFLS, flavonol synthase; FaGT1, anthocyanidin glucosyltransferase; FaGT2, (hydroxy)cinnamic acid and (hydroxy)benzoic acid glucosyltransferase; FaPAL, phenylalanine ammonia lyase. Reactions that have not been fully elucidated are indicated with dotted lines.
transcriptional data (de Luis Balaguer and Sozzani, 2017). The genes selected for the analysis corresponded to 685 genes that were significantly differentially expressed in the receptacle of the FaMADS9-silenced fruits at both the white and red stages (Table S2). To infer the relationships among these genes, a transcriptional data set obtained from RNAseq of ripening receptacles at four stages (green, white, turning and red; Sánchez-Sevilla et al., 2017) was used. Previous to the analysis by GENIST, a clustering step was performed on the 685 selected genes using the RNAseq data of the FaMADS9-silenced receptacles (Table S1). The GENIST output is shown in Table S15. FaMADS9 is included in one of the five main clusters, which predicted 44 targets (Table S15). Analysis of the FaMADS9 subnetwork (Figure 6a) shows connections to four regulatory genes, namely FvH4_7g12810 (TCP9), FvH4_2g11150 (GET7), FvH4_7g01060 (ZFN2) and FvH4_5g17780 (KAN1), with the last two exhibited a two-way arrangement. FaMADS9 shared 27 targets with KAN1, including GET7, and 17 targets with ZFN2 (Table S15). GO analysis of the inferred FaMADS9 targets showed a prevalence of genes associated with primary energetic metabolism (Table S16).

Identification of the FaMADS9-binding DNA sequence

MADS-box proteins are transcription factors that bind DNA via their MADS domains. All MADS proteins recognize a similar consensus sequence in their target genes (CC(A/T)G) that has been called the CArG box (A rich) (Pellegri et al., 1995; Santelli and Richmond, 2000). Although CArG boxes are similar, they are not identical, thus conferring specificity to different MADS transcription factors (Aerts et al., 2018). To determine the DNA sequence to which FaMADS9 binds, we employed a protein-binding microarray (PBM11) (Godoy et al., 2011). FaMADS9 cDNA was fused to MBP and used to hybridize a microarray that contained all possible combinations of 11-mer oligonucleotides. FaMADS9 bound the sequences containing CCAAAAAAT (Figure 6b) which is highly similar to a CArG.

Analysis of the 1-kb promoter of the F. vesca genes corresponding to the putative FaMADS9 targets inferred from the GRN showed that the FaMADS9 CArG box was present in 73% of the genes (Table S17). This value is significantly higher than that obtained for a random sample of F. vesca genes.

Discussion

Genetic mutants associated with tomato fruit development and ripening have facilitated the identification of numerous candidate genes that can be tested as functional homologues in other fruit species. These genes include the ripening-associated transcription factor RIN, which encodes a SEPALATA MADS-box gene (Vrebalov et al., 2002). The RIN transcription factor has long been believed to be necessary for the induction of ripening (Giovannoni, 2007; Vrebalov et al., 2002). However, a recent study based on an RIN knockout mutant demonstrated that tomato fruit ripening is initiated without RIN, even though RIN activity is required for the completion of ripening (Ito et al., 2017). In strawberry, the antisense down-regulation of FaMADS9, a putative orthologue of the RIN gene, resulted in drastic changes in fruit morphology and firmness, colour development, and gene expression, including the repression of three MADS genes (Seymour et al., 2011). Here, we complement this study with the generation and in-depth analysis of four independent FaMADS9-silenced strawberry transgenic lines. In our study, the specificity of the transgene silencing is supported by the fact that only the expression of FaMADS9 among the 34 MADS genes expressed during fruit development was down-regulated in red receptacles. The discrepancies found in the phenotype of transgenic fruits in relation to the previous work (Seymour et al., 2011) can be explained by the highly specific silencing of FaMADS9 achieved here, as supported by the RNAseq data. Silencing of FaMADS9 in the previous work (Seymour et al., 2011) was performed by 5’-antisense down-regulation, and in addition to FaMADS9, three MADS-box genes were down-regulated (FvH4_4g23530, FvH4_6g37880 and FvH4_7g12670). Here, we found that there was no change in the expression of the FvH4_4g23530 and FvH4_7g12670 genes, while the gene FvH4_6g37880 was up-regulated. This gene corresponds to a SHATTERPROOF-like gene that is involved in some processes associated with strawberry fruit ripening (Daminato et al., 2013). Thus, differences in the off-target or downstream effects of the silencing process, as well as in the cultivars, could explain the discrepancies between the two studies.

FaMADS9 is involved in specific developmental processes at the green stage

A previous study of FaMADS9 expression in strawberry fruits showed the highest expression in the receptacles, with peaks observed at the green and red stages and diminished values observed at the white and turning stages (Sánchez-Sevilla et al., 2017). The generation of FaMADS9-specific silenced lines and exhaustive analysis of the transcriptomic and metabolic changes in transformed receptor have allowed the identification of the developmental processes in which this gene is involved. The elevated number of differentially expressed genes, 2670 genes at the white stage and 1943 at the red stage, is indicative of the central role played by this transcription factor in the growth and ripening of the receptacles of the strawberry fruit. Global analysis of the transcriptomic and metabolomic data shows that, as expected from the FaMADS9 expression pattern in the fruit, significant changes occurred at the green and red stages.

The green stage is characterized by active cell division that must be supported by the continuous supply of building blocks for the cell structure and composition and the activity of biosynthetic processes. Thus, it has been reported that in strawberry, starch accumulates extremely early in the fruit formation process, while starch degradation predominates during fruit growth and development (Souleyre et al., 2004). These changes in starch levels are correlated with the dramatic decrease in the levels of maltose and isomaltose, the products of starch degradation, during receptacle ripening (Fait et al., 2008). Our finding of altered levels of maltose and isomaltose in the FaMADS9-silenced fruits could indicate that the silencing of this gene disrupts the readiness of the fruit to degrade starch in order to fuel growth and ripening. This finding is consistent with earlier work on a tomato rin mutant, which indicated the importance of RIN in starch synthesis during early fruit development (Osorio et al., 2011). The changes reported here for the galactonic acid-1,4-lactone content are also indicative of the involvement of FaMADS9 in the growth processes occurring in green receptacles. Strawberry fruit is rich in L-ascorbic acid, which is feasibly involved in the maintenance of the redox buffering capacity during developmental processes (Foyer, 2015). In the green receptacle, ascorbate is likely synthesized via the L-galactose pathway (Cruz-Rus et al., 2011). Although we were unable to detect L-ascorbic acid in the primary chromatograms from the red
receptacle, we found a significant decrease in the level of galactonic acid-1,4-lactone, the final precursor of the biosynthesis pathway in the transgenic green receptacles. Additionally, flavonoids are actively synthesized in strawberry fruits with a stage-specific pattern, with proanthocyanidins and flavonol derivatives being highly abundant at the green stage (Fait et al., 2008). Our in-depth analysis of secondary metabolites in the green receptacles of the silenced lines showed significant changes in these compounds. Taken together, these results reinforce the previously postulated view that the \( \text{FaMADS9} \) gene plays a crucial regulatory role in early fruit development (Seymour et al., 2011).

Whole-transcriptome analysis facilitates the elucidation of the underlying molecular mechanisms supporting the stage and composition of the sample under study. Although our RNAseq analysis was not performed in green receptacles, the results obtained in white receptacles might be indicative of the transcript levels at the green stage. The white stage of strawberry fruit represents the final phase of the growing period and is preliminary to the ripening period. MapMan analysis of the enriched categories within the differentially expressed genes at the white stage also supports the role indicated above for \( \text{FaMADS9} \) at the early developmental stage, which is the case for the highly represented UDP-glucosyl/glucuronosyl transferases. These enzymes are involved in the synthesis of a great diversity of substrates (Caputi et al., 2012), with some of these enzymes participating in the synthesis of structural glucans (Iwai et al., 2002). Additionally, the number of intrinsic membrane proteins down-regulated in the transgenic receptacles delimits the function of \( \text{FaMADS9} \). The relationship between water movement and fruit growth and ripening has been widely studied (Fouquet et al., 2008). Aquaporins belong to the family of integral membrane channel proteins and play an important role in water homeostasis in plants. In strawberry fruit, it has been reported that a \( \text{FaNIP1-1} \) gene, positively regulated by abscisic acid and negatively regulated by auxins, is active in water transport in the fruit (Molina-Hidalgo et al., 2015). The only \( \text{NIP1-1} \) gene that was differentially expressed in the present study was up-regulated at the white stage. Transcription analysis in the white receptacles would also explain the decrease in the level of galactonic acid-1,4-lactone, the final intermediate in the ascorbate biosynthesis pathway, in the transgenic green receptacles. This decrease was correlated with down-regulation in the expression of the mannose-1-phosphate guanylyltransferase (\( \text{VTC1} \)) and GDP-L-galactose phosphorylase (\( \text{VTC2} \)).

![Figure 6](a) Representation of the \( \text{FaMADS9} \) subnetwork within the gene regulatory network (GRN). Node sizes reflect the number of targets that are inferred. (b) The consensus CArG sequence of \( \text{FAmADS9} \) binding to DNA.
genes, which encode key regulatory enzymes of ascorbate biosynthesis (Bulley and Laing, 2016).

**Effects of FaMADS9 on specific processes associated with receptacle ripening**

One of the most evident ripening-related changes in strawberry fruit is the progressive and extensive loss of firmness, resulting in part from cell wall disassembly (Brummell, 2006; Vicente et al., 2007). In strawberry, these processes were not altered after FaMADS9 silencing because fruit firmness was not changed in transgenic fruits, nor was the expression of cell wall degrading enzymes such as PLA, PLB and RGlyase in the red stage (Benitez-Burraco et al., 2003; Jiménez-Bermúdez et al., 2002; Molina-Hidalgo et al., 2013; Quesada et al., 2009). Up-regulation of PG1 and PG2 genes has no apparent effect on the firmness of the red fruits. This finding excludes a role for FaMADS9 in normal ripening-related cell wall degradation. However, this result was in direct contrast to the rin tomato mutant, which exhibited down-regulation of ripening-related wall metabolism genes (Ostoró et al., 2011).

Formation of the cuticle, which is the lipophilic layer that covers the outer epidermal cell wall of the aerial parts of higher plants (Jeffree, 2006), has been described as an important process during fruit ripening (Jeffree, 2006; Nawrath, 2006). In tomato fruit, the cuticle of the rin mutant has been described to have a lipid composition that differs from that of normal fruit, which impacts development throughout ripening (Kosma et al., 2010). Similarly, we found differences in the lipid composition of the cuticular wax of FaMADS9-silenced fruit. Repression of the regulatory genes SHN1/MVIN1 and SHNE3, the latter of which is the closest homolog to the tomato SISHN3 (Shi et al., 2013), in the transgenic receptacles would indicate a role for FaMADS9 in this key aspect of the fruit ripening process. Moreover, the putative cutin synthase gene FcCD1, mostly expressed in the epidermal layer, was down-regulated in the transgenic receptacle, which suggested that cutin composition and structure must also be altered.

With respect to the changes in flavonoid content in the FaMADS9-silenced receptacles, we observed changes in metabolites that were limited to the most abundant condensed tannins, flavonols and anthocyanins. Some metabolic differences were accompanied by corresponding transcriptional changes. Thus, changes in the levels of flavonols and cyanidin-hexoses were correlated with the down-regulation of the flavonoid 3'-hydroxylase gene (F3’H) in FaMADS9-silenced receptacles. However, changes in the levels of other metabolites were not correlated with changes in the levels of transcripts of genes encoding metabolic enzymes. This finding may suggest that in these cases, the metabolic changes are likely due to the adjustment of metabolic fluxes in response to primary changes in other steps of the pathway. Secondary redirection of the flavonoid metabolism in strawberry fruits has been reported after a primarily induced change in the expression of a specific gene (Fischer et al., 2013).

The involvement of FaMADS9 in the regulatory system of flavonoid metabolism in the receptacles is supported by the number of TFs, previously reported to participate in the control of the pathway, the expression of which was altered in the FaMADS9-silenced lines. These genes included MYB10 (FvH4_1g22020), PCF1 (FvH4_6g16170), DIV (FvH4_6g11950), ZFN1 (FvH4_6g00570) and ZFN2 (FvH4_7g01060) (Pillet et al., 2015). The complex interaction among these regulatory genes in relation to flavonoid biosynthesis, as well as other developmental changes in ripening receptacles, requires the global analysis of available data sets associated with the process.

**Involvement of hormones in the action of FaMADS9 on strawberry receptacle ripening**

The numerous metabolic and transcriptional changes found after the specific silencing of FaMADS9, despite the apparent lack of visual changes, point to an involvement of this gene product in the regulatory machinery governing strawberry fruit ripening. Most of the hormones have been reported to participate in strawberry fruit development and ripening (Chai et al., 2013; Csukasi et al., 2011; Estrada-Johnson et al., 2017; Jia et al., 2011; Merchante et al., 2013; Mezzetti et al., 2004), and they are expected to play a main role in the regulation of the process. However, the interplay between hormones and regulatory genes has scarcely been addressed (Csukasi et al., 2012; Vállarino et al., 2015). Here, we found that silencing of FaMADS9 causes changes in the expression of an elevated number of ARFs but also a drastic reduction in the expression of ABA-synthesizing genes such as FaNCED1, FaNCED2 and FaNCED3 in the transgenic receptacles, and a subsequent decrease in ABA content. Silencing of FaNCED1 and FaNCED2 was observed in FaGAMYB-silenced receptacles (Vállarino et al., 2015), as well as reduced levels of ABA in the transgenic tissue. Notably, the ASR gene (FvH4_2g31410), which is involved in the ABA transduction pathway, was up-regulated in red receptacles. This gene has been reported to play a key role in strawberry fruit ripening (Jia et al., 2016). On the other hand, the ABA-negative regulator SnRK2.6 (FvH4_2g06910), which is also involved in the regulation of strawberry fruit ripening (Han et al., 2015), was up-regulated in white receptacle. All these results point to a drastic change in ABA signalling in the FaMADS9-silenced receptacle from early stages of fruit development. Particularly interesting is the SHP gene, the only MADS up-regulated in the transgenic receptacle, as this gene has been reported to play a main role in strawberry fruit ripening, and the expression of this gene is antagonistically regulated by auxin and ABA (Daminato et al., 2013).

The inference of gene regulatory networks (GRNs) has been proposed as a method for identifying candidate genes from large-scale data sets. This method uses transcriptional data as input and produces an inferred network showing the most likely regulatory pathways of the genes of interest based on the data. The availability of time course data allowed us to infer directed networks based on dynamic Bayesian networks (de Luis Balaguer et al., 2017) that infer statistical dependence among selected genes. Here, applied to the DE genes in the white and red stages of FaMADS9-silenced receptacles, this method provided a subnetwork centred around FaMADS9 with connections to different regulatory genes. The presence of the FaMADS9 DNA-binding sequence in the promoters of the corresponding F. vesca genes further supports to a possible direct interaction between FaMADS9 and the inferred targets, some of them consistent with the additional information provided in the present publication. Thus, the KAN1 gene has been reported to act in Arabidopsis as a transcriptional repressor of genes in the auxin action pathway (Huang et al., 2014), whereas the ZFN2 and DIV genes have been linked to anthocyanin biosynthesis in strawberry fruit (Pillet et al., 2015). Other predicted targets, such as GTE7, belong to a family of transcription factors that mediate ABA and sugar response in Arabidopsis (Misra et al., 2018).
In summary, our transcriptomic and metabolomic studies on the receptacles of strawberry transgenic lines, in which the SEPALLATA MADS-box gene FaMADS9 was silenced, reveal that this gene is integrated into the regulatory network controlling the ripening of this non-climacteric fruit. Furthermore, this role was extended to the growth of the receptacles at early developmental stages. As was recently reported for the putative RIN orthologue in tomato fruit, FaMADS9 is not a general regulator but is involved in specific developmental changes that occur during strawberry ripening, some of which are relevant for fruit quality parameters such as the Brix index, the flavonoid content and the composition of the cuticle waxes. Additionally, we were able to draw several important conclusions regarding transcriptomic/metabolomic regulation during strawberry ripening, thus providing new insights into the regulatory network underlying strawberry fruit ripening. However, the inference of the GRN and the presence of FaMADS9 DNA-binding sequence in the inferred targets do not proof a direct interaction between them. Thus, a transactivation assay should confirm this possibility.

**Experimental Procedures**

**Plant material and sampling**

Silencing of FaMADS9 (GenBank AF484683) was performed in Fragraaria x ananassa Duch cv. Camarosa. Given the high sequence similarity between different members of the MADS-box gene family, the fragment chosen for silencing was located in the C-terminal domain, which is the most divergent between MADS genes (primers listed in Table S18). The construct for the post-transcriptional silencing (FaMADS9-RNAi) was generated from a 241 bp fragment of FaMADS9 from cv. Camarosa and cloned into pHANNIBAL. Then, it was cut with SpeI/SalI and cloned in XbaI/Sacl sites of pBINplus binary vector. Strawberry plants were transformed as previously described by El-Mansouri et al., (1996). The fruits were harvested at three different developmental stages: green, G; white, W; and red, R. Transcriptome and metabolomic analyses were performed in three separate pools of 20–25 fruits each. Each pool was from three different plants. These biological replicates came from 9 transgenic and wild-type plants (all of which were grown in the greenhouse; the genotypes were randomly distributed). All fruits were frozen immediately in liquid nitrogen, and achenes were removed using a scalpel on frozen fruits.

**RNA extraction and transcriptome analysis by RNAseq**

Total RNA was extracted as previously reported (Vallarino et al., 2015). RNA integrity was verified using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and RNA integrity number (RIN) values were >8 for all biological replicates. Expression analysis by RNAseq of transgenic lines (L6 and L7) and WT was performed in triplicate RNA from receptacle tissue in two development stages (white and red). Libraries preparation, sequencing and mapping were performed as previously described (Sánchez-Sevilla et al., 2017). Normalized RNAseq fragment counts were used to measure the relative abundances of transcripts expressed as fragments per kilobase of exon per million fragments mapped (FPKM). For assignment of functional gene predictions, MapMan ‘BINs’ (Lohse et al., 2014) and open-source F. vesca gene ontology (GO) annotation (Darwish et al., 2015; Edger et al., 2018; Shulaev et al., 2011) were used. RNAseq raw data are available under http://www.ebi.ac.uk/ena/data/view/PRJEB32676.

**Gene expression analysis by quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted as described previously for the transcriptome analysis. These biological replicates were different from those used for transcriptome analysis. Expression data were normalized to the reference genes actin and glyceraldehyde-3-phosphate dehydrogenase (GADPH), with comparable results. Values showed in this publication are normalized to GADPH (Salvatierra et al., 2010). Primers are listed in Table S18.

**Extraction and analysis of polar metabolites using GC-MS and UPLC-Orbitrap-MS/MS**

Primary metabolite profiles and ABA were obtained by GC-time of flight-(TOF)-MS from the same material as used for transcriptome analysis. Metabolite extraction, derivatization and analysis were determined as described by Osorio et al. (2012). Secondary metabolite analysis system was performed using method described by Vallarino et al. (2018) by Waters Acquity UPLC. Full documentation of metabolite profiling data acquisition and interpretation is provided in Table S14.

**Wax analysis**

A protocol based on Yeats et al. (2012b) was used for wax analysis.

**DNA-binding domain**

Identification of the FaMADS9 DNA-binding motif and statistical analysis was performed as in Berger and Bulyk (2009) with the exception that the cDNA of FaMADS9 was fused to MBP (Maltose binding protein). The consensus logo was done with the use of the enoLOGOS server (http://agavulin.ccbb.pitt.edu/cgi-bin/eno logos/enoLogos.cgi).

Promoter analysis for the detection of putative transcription factor binding sites was performed on the PlantPAN3.0 resource (http://plantpan.itps.ncku.edu.tw/index.html), developed by Chow et al. (2019).

**Gene regulatory network**

To deduce the GRN, a set of 685 genes that were significantly differentially expressed in the receptacle of the FaMADS9-silenced fruits compared to the untransformed fruits, both at white and red stages, were selected. For this set of genes, a computational pipeline (GENIST) (de Luis Balaguer et al., 2017) was used to infer their relationships from a combination of spatial (achen, receptacle and leaf) and temporal (green; white; turning; red) transcriptional data obtained by RNAseq (Sánchez-Sevilla et al., 2017). Clustering of genes before the inference step by GENIST has been shown to improve GENIST performance (de Luis Balaguer et al., 2017), since it reduces the complexity of the inference steps for large networks. Therefore, a clustering step was performed previous to inference by GENIST. The clustering data here used were the RNAseq expression data obtained from the receptacle of control and FaMADS9-silenced fruits, at white and red stages (Table S1). Application of GENIST was performed as previously reported (de Luis Balaguer et al., 2017).

**Acknowledgements**

This work was supported by grants BIO2013-44199-R, AGR12-40066-C02-02, and RTI2018-099797-B-100 (MINECO, Spain), and the European Union’s H2020 Programme (GoodBerry; grant
number 679303) and ERC-2014-StG638134. DP and SO acknowledge the support by Spanish Ministry of Science and Innovation (Ramón and Cajal contracts, RYC2011-09170 and RYC2013-12699).

Conflicts of interest
The authors declare no conflict of interest.

Author contributions
V.V. and S.O. coordinated the analysis of the data and prepared the manuscript. J.G.V. and S.O. performed the evaluation, characterization and metabolic analysis of the transgenic lines. C.M cloned the gene and made the initial expression analysis. C.M and A.C. made the FaMADS9-RNAi silencing constructs and generated the transgenic lines. C.M., A.C and M.T.A performed the preliminary characterization of the transgenic lines. A.V. made the expression analyses that involved different parts of the receptacle. I.A., D.P., MAB and J.F.S-S performed the analysis of the RNASEq data. M.A.d.L.B., R.S. and V.V. made the GRN. L.W. and A.R.F performed the metabolomic analysis. R.S. performed the DNA-binding domain experiment. J.J.G. made the RNASEq experiment. All authors participated and reviewed the writing of the manuscript.

References
Aerts, N., de Brujin, S., van Mourik, H., Angenent, G.C. and van Dijk, A.D.J. (2018) Comparative analysis of binding patterns of MADS-domain proteins in Arabidopsis thaliana. BMC Plant Biol. 18, 131.
Aharoni, A. and O’Connell, A.P. (2002) Gene expression analysis of strawberry achene and receptacle maturation using DNA microarrays. J. Exp. Bot. 53, 2073–2087.
Aharoni, A., De Vos, C.H., Wein, M., Sun, Z., Greco, R., Kroon, A., Møl, J.N. et al. (2001) The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. Plant J. 28, 319–332.
Aharoni, A., Dietz, S., Letter, R., Thoenes, E., van Arkel, G. and Pereira, A. (2004) The SHINE clade of AP2 domain transcription factor activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. Plant Cell, 16, 2463–2480.
Araguz, I., Osorio, S., Hoffmann, T., Rambla, J.L., Medina-Escobar, N., Granell, A., Botella, M.A. et al. (2013) Eugenol production in achenes and receptacles of strawberry fruits is catalysed by synthases exhibiting distinct kinetics. Plant Physiol. 163, 946–958.
Benitez-Burraco, A., Blanco-Portales, R., Redondo-Nevado, J., Bellido, M.L., Moyano, E., Caballero, I.L. and Muñoz-Blanco, I. (2003) Cloning and characterization of two ripening-related strawberry (Fragaria x ananassa cv. Chandler) pectate lyase genes. J. Exp. Bot. 54, 633–645.
Berger, M.F. and Bulyk, M.L. (2009) Universal protein-binding microarrays for the comprehensive characterization of the DNA-binding specificities of transcription factors. Nat. Protoc. 4, 393–411.
Bernard, A. and Joubit, J. (2013) Arabidopsis cuticular waxes: Advances in synthesis, export and regulation. Prog. Lipid. Res. 52, 110–129.
Brummell, D.A. (2008) Cell wall disassembly in ripening fruit. Funct. Plant Biol. 35, 103–119.
Bulley, S. and Laing, W. (2016) The regulation of ascorbate biosynthesis. Curr. Opin. Plant Biol. 33, 15–22.
Caputi, J., Malnoy, M., Gorenzykin, V., Nikiforova, S. and Martens, S. (2012) A genome-wide phylogenetic reconstruction of family I UDP-glycosyltransferases revealed the expansion of the family during the adaptation of plants to life on land. Plant J. 69, 1030–1042.
Chai, Y.M., Jia, H.F., Li, C.L., Dong, Q.H. and Shen, Y.Y. (2011) FaFYR1 is involved in strawberry fruit ripening. J. Exp. Bot. 62, 5079–5089.

FaMADS9 gene influences strawberry ripening metabolism

Chai, Y.M., Zhang, Q., Tian, L., Li, C.L., Xing, Y., Qin, L. and Shen, Y.Y. (2013) Brassinosteroid is involved in strawberry fruit ripening. Plant Growth Regul. 69, 63–69.
Chow, C.N., Lee, T.Y., Hung, Y.C., Li, G.Z., Tseng, K.C., Liu, Y.H., Kuo, P.L. et al. (2019) PlantPAN3.0: a new and updated resource for reconstructing transcriptional regulatory networks from ChIP-seq experiments in plants. Nucleic. Acids Res. 47(D1), D1155–D1163.
Concha, C.M., Figueroa, N.E., Poblete, L.A., Ohiate, F.A., Schwab, W. and Figueroa, C.R. (2013) Methyl jasmonate treatment induces changes in fruit ripening by modifying the expression of several ripening genes in Fragaria chiloensis fruit. Plant Physiol. Biochem. 70, 433–444.
Cruz-Rus, E., Amaya, I., Sánchez-Sevillano, J.F., Botella, M.A. and Valpuesta, V. (2011) Regulation of L-ascorbic acid content in strawberry fruit. J. Exp. Bot. 62, 4191–4201.
Cuska, F., Osorio, S., Gutierrez, J.R., Kitamura, J., Gaivaloso, P., Nakajima, M., Fernie, A.R. et al. (2011) Gibberellin biosynthesis and signalling during development of the strawberry receptacle. New Physiol. 191, 376–390.
Cuska, F., Donaire, L., Casaral, A., Martínez-Priego, L., Botella, M.A., Medina-Escobar, N., Llave, C. et al. (2012) Two strawberry mirt159 family members display developmental specific expression patterns in the fruit receptacle and cooperatively regulate Fa-GAMYB. New Physiol. 195, 47–57.
Daminato, M., Guzzo, F. and Casadoro, G. (2013) A SHATTERPROOF-like gene controls ripening in non-climacteric strawberries, and auxin and abscisic acid antagonistically affect its expression. J. Exp. Bot. 64, 3775–3786.
Danish, O., Shahari, R., Liu, Z., Slavin, J.P. and Alkilany, N.W. (2015) Re-annotation of the woodland strawberry (Fragaria vesca) genome. BMC Genom. 16, 29.
Domínguez, E., Heredia-Guerrero, J.A. and Heredia, A. (2015) Plant cutin genesis: unanswered questions. Trends Plant Sci. 20, 551–558.
Edger, P.P., VanBuren, R., Cole, M., Poorten, T.J., Wai, C.M., Niederhuth, C.E., Alger, E.I. et al. (2018) Single-molecule sequencing and optical mapping yields an improved genome of woodland strawberry (Fragaria vesca) with chromosome-scale contiguity. GigaScience, 7, 1–7.
Elitzur, T., Vrebalo, J., Giovannoni, J.J., Goldschmidt, E.E. and Friedman, H. (2010) The regulation of MADS-box gene expression during ripening of banana and their regulatory interaction with ethylene. J. Exp. Bot. 61, 1523–1535.
El-Mansouri, I., Mercado, J.A., Valpuesta, V., López-Aranda, J.M., Piego, F. and Quesada, M.A. (1996) Shoot regeneration and Agrobacterium mediated transformation of Fragaria vesca L. Plant Cell Rep. 15, 642–646.
Estrada-Johnson, E., Cuska, F., Pizano, C.M., Vallano, I.G., Krysko, Y., Vioque, A., Merchante, C. et al. (2017) Transcriptome analysis in strawberry fruits reveals active auxin biosynthesis and signaling in the ripe receptacle. Front. Plant Sci. 8, 889.
Falt, A., Hanhineva, K., Beleggia, R., Dai, N., Rogachev, I., Nikiforova, V.I., Fernie, A.R. et al. (2008) Reconfiguration of achene and receptacle metabolic networks during strawberry fruit development. Plant Physiol. 148, 730–750.
Fischer, T.C., Mirbath, B., Rentsch, J., Sutter, C., Ring, L., Flachowsky, H., Habegger, R. et al. (2013) Premature and ectopic anthocyanin formation in strawberry (Fragaria x ananassa) plants. New Physiol. 201, 440–451.
Fouquet, R., León, C., Ollat, N. and Barrieu, F. (2008) Identification of grapevine aquaporins and expression analysis in developing berries. Plant Cell Rep. 27, 1541–1550.
Foyer, C.H. (2015) Redox homeostasis: Opening up ascorbate transport. Nat. Plants, 1, 14012.
Giovannoni, J.J. (2007) Fruit ripening mutants yield insights into ripening control. Curr. Opin. Plant Biol. 10, 283–289.
Godoy, M., Franco-Zorrilla, J.M., Pérez-Pérez, J., Oliveros, J.C., Lorenzo, O. and Solano, R. (2011) Improved protein-binding microarrays for the identification of DNA-binding specificities of transcription factors. Plant J. 66, 700–711.
Han, J.Y., Kim, J.M., Ban, Y.W., Hwang, H.S. and Choi, Y.E. (2013) The involvement of β-amyrin 28-oxidase (CYP716A52v2) in oleanane-type ginsenoside biosynthesis in Panax ginseng. Plant Cell Physiol. 54(12), 2034.2046.
Han, Y., Dang, R., Li, J., Jiang, Z., Zhang, N., Jia, M., Wei, L. et al. (2015) SUCCROSE NONFERMENTING1-RELATED PROTEIN KINASE2.6, an ortholog of OPEN STOMATA1, is a negative regulator of strawberry fruit development and ripening. Plant Physiol. 167, 915–930.

© 2019 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 18, 929–943
Medina-Puche, L., Molina-Hidalgo, F.J., Boersma, M., Schuurink, R.C., López-Vidriero, I., Solano, R., Franco-Zorrilla, J.M. et al. (2015) An R2R3-MYB transcription factor regulates eugenol production in ripe strawberry fruit receptacles. Plant Physiol. 168, 598–614.

Merchant, C., Vallarino, J.G., Osorio, S., Aragüez, I., Villarreal, N., Ariza, M.T., Martínez, G.A. et al. (2013) Ethylene is involved in strawberry fruit ripening in an organ-specific manner. J. Exp. Bot. 64(14), 4421–4439.

Mezzetti, B., Landi, L., Pandolfini, T. and Spena, A. (2004) The deH8-IAAaM auxin-synthesizing gene increases plant fecundity and fruit production in strawberry and raspberry. BMC Biotechnol. 4, 4.

Mira, A., McKnight, T.D. and Marndadi, K.K. (2018) Bromodomain proteins GTE9 and GTE11 are essential for specific BT2-mediated sugar and ABA responses in Arabidopsis thaliana. Plant Mol. Biol. 96(4–5), 393–402.

Molina-Hidalgo, F.J., Franco, A.R., Villalero, C., Medina-Puche, L., Mercado, J.A., Hidalgo, M.A., Monfort, A. et al. (2013) The strawberry (Fragaria x ananassa) fruit-specific rhamnogalacturonate lyase 1 (FaGLyase1) gene encodes an enzyme involved in the degradation of cell-wall middle lamellae. J. Exp. Bot. 64, 1471–1483.

Molina-Hidalgo, F.J., Medina-Puche, L., Gelis, S., Ramos, J., Sabir, F., Soveral, G., Pritza, C. et al. (2015) Functional characterization of FaHPI1, a gene, ripening and receptacle-specific aquaticin in strawberry fruit. Plant Sci. 238, 198–211.

Molina-Hidalgo, F.J., Medina-Puche, L., Cañete-Gomez, C.J., Franco-Zorrilla, J.M., López-Vidriero, I., Solano, R., Caballero, J.L. et al. (2017) The fruit-specific FaDF02 transcription factor regulates the production of eugenol in ripe fruit receptacle. J. Exp. Bot. 68, 4529–4543.

Mukkun, L. and Singh, Z. (2009) Methyl jasmonate plays a role in fruit ripening of ‘Pajaro’ strawberry through stimulation of ethylene biosynthesis. Sci. Hortic. 123, 5–10.

Nawrath, C. (2006) Unraveling the complex network of cuticular structure and function.Curr. Opin. Plant Biol. 9, 281–287.

Nitsch, J. (1950) Growth and morphogenesis of the strawberry as related to auxin. Am. J. Bot. 37, 211–215.

Osorio, S., Bombarely, A., Giavalisco, P., Usadel, B., Stephens, C., Aragüez, I., Medina-Escobar, N. et al. (2011) Demethylolation of oligogalacturonides by FaPE1 in the fruits of the wild strawberry Fragaria vesca triggers metabolic and transcriptional changes associated with defense and development of the fruit. J. Exp. Bot. 62, 2855–2873.

Osorio, S., Do, P.T. and Ferrie, A.R. (2012) Profiling primary metabolites of tomato fruit with gas chromatography/mass spectrometry. Methods Mol. Biol. 860, 101–109.

Pellegrini, L., Tan, S. and Richmond, T.J. (1995) Structure of serum response factor core bound to DNA. Nature, 376, 490–498.

Perkins-Veazie, P. (1995) Growth and ripening of strawberry fruit. Hortic. Rev. 17, 267–297.

Pillet, J., Yu, H.-W., Chambers, A.H., Whitaker, V.M. and Foita, K.M. (2015) Identification of candidate flavonoid pathway genes using transcriptome correlation network analysis in ripe strawberry (Fragaria × ananassa) fruits. J. Exp. Bot. 66, 4455–4467.

Quedada, M.A., Blanco-Porlales, R., Posé, S., García-Gago, J.A., Jiménez-Bermúdez, S., Muñoz-Serrano, A., Caballero, J.L. et al. (2009) Antisense down-regulation of the FaPG1 gene reveals an unexpected central role for polygalacturonase in strawberry fruit softening. Plant Physiol. 150, 1022–1032.

Salvatierra, A., Pimentel, P., Moya-Leon, M.A., Calgarí, P.D.S. and Herrera, R. (2010) Comparison of transcriptional profiles of flavonoid genes and anthocyanin contents during fruit development of two botanical forms of Fragaria chiloensis spp. chiloensis. Phytomorph. 71, 1839–1847.

Sánchez-Sevill, J.F., Vallarino, J.G., Osorio, S., Bombarely, A., Posé, D., Merchante, C., Botella, M.A. et al. (2017) Gene expression atlas of fruit ripening and transcriptional assembly from RNA-seq data in octoploid strawberry (Fragaria × ananassa). Sci. Rep. 7, 13737.

Sanetti, E. and Richmond, T.J. (2000) Crystal structure of MEF2A core bound to DNA at 1.5 Å resolution. J. Mol. Biol. 29, 437–449.

Schaart, J., Dubos, C., Romero de la Fuente, I., van Houwelingen, A., De Vos, R., Jonker, H., Xu, W. et al. (2013) Identification and characterization of MYB-BHLH-WD40 regulatory complexes controlling proanthocyanidin biosynthesis in strawberry (Fragaria x ananassa) fruits. New Phytol. 197, 454–467.
Schulenburg, K., Feller, A., Hoffmann, T., Schecke, J.H., Martin, S. and Schwab, W. (2016) Formation of β-glucogallin, the precursor of ellagic acid in strawberry and raspberry. J. Exp. Bot. 67, 2299–2308.

Seymour, G.B., Ryder, C.D., Cevik, V., Hammond, J.P., Popovich, A., King, G.J., Vrebalov, J. et al. (2011) A SEPALATA gene is involved in the development and ripening of strawberry (Fragaria x ananassa Duch.) fruit, a non-climacteric tissue. J. Exp. Bot. 62, 1179–1188.

Shi, J.X., Adato, A., Alkan, N., He, Y., Lashbrook, J., Matas, A.J., Meir, S. et al. (2013) The tomato SISHNE1 transcription factor regulates fruit cuticle formation and epidermal patterning. New Phytol. 197, 468–480.

Shulaev, V., Sargent, D.J., Crowhurst, R.N., Mockler, T.C., Folkers, O., Delcher, A.L., Jaiswal, P. et al. (2011) The genome of woodland strawberry (Fragaria vesca). Nat Genet. 43, 109–116.

Souleyre, E.J.F., Taylor, M., Iannetta, P.P.M., Ross, H.A., Shepherd, L.V.T., Hancock, R., Viola, R. et al. (2004) Carbohydrate metabolism in ripening strawberry fruit. Physiol. Plant. 121, 369–376.

Tadello, A., Pavanello, A., Zanin, D., Caporal, E., Colombo, L., Rotino, G.L., Trainotti, L. et al. (2009) A PLENA-like gene of peach is involved in carpel formation and subsequent transformation into a fleshy fruit. J. Exp. Bot. 60, 651–661.

Thimm, O., Blässing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., Selbig, J. et al. (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J. 37, 914–939.

Tranberger, T.J., Dussert, S., Joët, T., Argout, X., Summo, M., Champion, A., Cros, D. et al. (2011) Regulatory mechanisms underlying oil palm fruit mesocarp maturation, ripening, and functional specialization in lipid and carotenoid metabolism. Plant Physiol. 156, 564–584.

Vallarino, J.G., Osorio, S., Bombarely, A., Casaralí, A., Cruz-Ruí, E., Sánchez-Sevilla, J.F., Arnaya, I. et al. (2015) Central role of FaGAMYB in the transition of strawberry receptacle from development to ripening. New Phytol. 208, 482–496.

Vallarino, J.G., de Abreu e Lima, F., Soria, C., Tong, H., Pott, D.M., Willmitzer, L., Fernie, A.R. et al. (2018) Genetic diversity of strawberry germplasm using metabolomic biomarkers. Sci. Rep. 8, 14386.

Valpuesta, V. and Botella, M.A. (2004) Biosynthesis of L-ascorbic acid in plants. New pathways for an old antioxidant. Trends Plant Sci. 9, 573–577.

Vicente, A.R., Salidé, M., Rose, J.K.C. and Labavitch, J.M. (2007) The linkage between cell wall metabolism and the ripening-associated softening of fruits: looking to the future. J. Sci. Food Agric. 87, 1435–1448.

Vrebalov, J., Ruzinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W. et al. (2002) A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. Science, 296, 343–346.

Yeats, T.H., Martin, L.B.B., Viart, H.M.F., Isaacson, T., He, Y., Zhao, L., Matas, A.J. et al. (2012a) The identification of cutin synthase: formation of the plant polyester cutin. Nat. Chem. Biol. 8, 609–611.

Yeats, T.H., Buda, G.J., Wang, Z., Chehanovsky, N., Moyle, L.C., Jetter, R., Schaffer, A.A. et al. (2012b) The fruit cuticle of wild tomato species exhibit architectural and chemical diversity, providing a new model for studying the evolution of cuticle function. Plant J. 69, 655e666.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Expression by quantitative real-time PCR (qRT-PCR) of four MADS genes in the receptacle of FaMADS9-silenced lines at white and red stages.

Figure S2 Changes in primary metabolism in the receptacle of FaMADS9-silenced lines at three ripening stages.

Figure S3 Gene expression of genes involved in waxes and cutin biosynthesis.

Table S1 RNAseq full table.

Table S2 Differentially expressed genes in receptacle of WT and FaMADS9-silenced lines (L6 and L7) at white and red stage by RNAseq.

Table S3 Expression analysis of Fragaria MADS genes by RNAseq in receptacle of WT and FaMADS9-silenced lines (L6 and L7) at white and red stage.

Table S4 List of enriched genes from Mapman categories from Figure 2.

Table S5 Expression analysis of Fragaria auxin-related genes by RNAseq in receptacle of WT and FaMADS9-silenced lines (L6 and L7) at white and red stage.

Table S6 Expression analysis of Fragaria NIP, PIP, TIP, and SLIP genes by RNAseq in receptacle of WT and FaMADS9-silenced lines (L6 and L7) at white and red stage.

Table S7 Relative metabolite content in fruits of FaMADS9-silenced plants.

Table S8 Expression analysis of Fragaria ß-amylases, and isoamylases genes by RNAseq in receptacle of WT and FaMADS9-silenced lines (L6 and L7) at white and red stage.

Table S9 Expression analysis of Fragaria L-ascorbic acid biosynthesis genes by RNAseq in receptacle of WT and FaMADS9-silenced lines (L6 and L7) at white stage.

Table S10 Expression analysis of Fragaria cell wall-modifying genes by RNAseq in receptacle of WT and FaMADS9-silenced lines (L6 and L7) at white and red stage.

Table S11 Expression analysis of Fragaria wax metabolism-related genes by RNAseq in receptacle of WT and FaMADS9-silenced lines (L6 and L7) at white and red stage.

Table S12 ABA content of WT and FaMADS9-silenced red fruits.

Table S13 Tentatively identified metabolites in the UPLC-FT-ICR-MS and their abundance in fruits of FaMADS9-silenced plants.

Table S14 Expression analysis of Fragaria coumarate-coenzyme A ligase (CL) genes by RNAseq in receptacle of WT and FaMADS9-silenced lines (L6 and L7) at white and red stages.

Table S15 List of dynamic gene interactions.

Table S16 GO analysis of the FaMADS9 targets from Table S15.

Table S17 Analysis of the 1 Kb promoter of F. vesca genes corresponding to putative FaMADS9 targets inferred from the GRN (Table S15).

Table S18 List of primers.