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Article Title: A SEPALLATA gene is involved in the development and ripening of strawberry (*Fragaria3ananassa* Duch.) fruit, a non-climacteric tissue

Year of publication: 2010

Link to published article:

<http://dx.doi.org/10.1093/jxb/erq360>

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RESEARCH PAPER

A *SEPALLATA* gene is involved in the development and ripening of strawberry (*Fragaria* × *ananassa* Duch.) fruit, a non-climacteric tissue*

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Received 25 August 2010; Revised 8 October 2010; Accepted 11 October 2010

Abstract

Climacteric and non-climacteric fruits have traditionally been viewed as representing two distinct programmes of ripening associated with differential respiration and ethylene hormone effects. In climacteric fruits, such as tomato and banana, the ripening process is marked by increased respiration and is induced and co-ordinated by ethylene, while in non-climacteric fruits, such as strawberry and grape, it is controlled by an ethylene-independent process with little change in respiration rate. The two contrasting mechanisms, however, both lead to texture, colour, and flavour changes that probably reflect some common programmes of regulatory control. It has been shown that a *SEPALLATA*(*SEP*)4-like gene is necessary for normal ripening in tomato. It has been demonstrated here that silencing a fruit-related *SEP1/2*-like (*FaMADS9*) gene in strawberry leads to the inhibition of normal development and ripening in the petal, achene, and receptacle tissues. In addition, analysis of transcriptome profiles reveals pleiotropic effects of *FaMADS9* on fruit development and ripening-related gene expression. It is concluded that *SEP* genes play a central role in the developmental regulation of ripening in both climacteric and non-climacteric fruits. These findings provide important information to extend the molecular control of ripening in a non-climacteric fruit beyond the limited genetic and cultural options currently available.

Key words: *Fragaria*, fruit development, ripening, *SEPALLATA* genes, strawberry.

Introduction

Fruits are reproductive organs unique to the Angiosperms that have evolved to promote seed dispersal. They are also one of the most important components of the human diet. Classically, two broad classes of fruits have been recognized. In climacteric fruits, for example, tomato (*Solanum lycopersicum*), banana (*Musa acuminata*), and melon (*Cucumis melo*), the onset of ripening is marked by increased ethylene synthesis and respiration. In non-climacteric fruits, for example, strawberry (*Fragaria* × *ananassa*), grape (*Vitis vinifera*), and citrus, these changes are not apparent (Giovannoni, 2004;

Seymour *et al.*, 1993). However, these distinct developmental programmes usually result in similar ripening-related changes including a loss of chlorophyll, the accumulation of carotenoids or anthocyanins, the production of volatiles, and cell wall disassembly.

The role of ethylene in inducing ripening in climacteric fruits has received substantial attention. The pathway for ethylene biosynthesis is now well established and many steps in the ethylene signal transduction pathway have been revealed (Alexander and Grierson, 2002). In stark contrast, almost

nothing is known about the molecular regulation of ripening in non-climacteric fruits (Giovannoni, 2004).

Studies on non-ripening mutants of tomato suggest that certain hitherto unsuspected developmental cues may control ripening. In tomato, the *ripening inhibitor (rin)* mutation exhibits pleiotropic effects in the fruits that fail to soften, lack a respiratory climacteric and associated ethylene evolution, pigment accumulation, and flavour or aroma development (Robinson and Tomes, 1968). Positional cloning has revealed that a *SEPALLATA (SEP)*-like MADS-box transcription factor (*LeMADS-RIN*), resides at the *rin* locus and the gene is expressed primarily in fruit tissues (Vrebalov *et al.*, 2002). The *LeMADS-RIN* protein contains consensus binding sites characteristic of the SEP proteins and *in vitro* recognizes similar DNA-binding sites to these transcription factors (Ito *et al.*, 2008). However, in *Arabidopsis SEP* genes are commonly associated with the regulation of floral organ identity suggesting that, in tomato fruit, *LeMADS-RIN* has evolved a different biological function. In strawberry, a non-climacteric fruit, it is shown that silencing a *SEP1/2* class gene in transgenic plants, which is expressed during normal fruit development and ripening, delays and modifies these important processes.

Materials and methods

Phylogenetic analysis

A phylogenetic tree was constructed based on predicted full-length protein sequences using MegAlign software (DNASStar) and aligned by ClustalW.

Transgenic strawberry lines

A 552bp antisense fragment from the 5' end of *FaMADS9 cDNA*, the putative strawberry orthologue (GenBank AF484683) of *LeMADS-RIN* described previously (Vrebalov *et al.*, 2002), was cloned in front of the CaMV35S promoter at the *KpnI/BamHI* multiple cloning site of the pBINPLUS1 plant transformation vector (Van Engelen *et al.*, 1995). Leaf discs of the cultivar Calypso were transformed and kanamycin-resistant plants selected as described previously by Woolley *et al.* (2001).

Tissue sampling

Individual receptacles (minus achenes) were powdered in liquid N₂ and subsamples were taken for the determination of chlorophyll, anthocyanin, and *RIN* expression.

Determination of chlorophyll, anthocyanin, and fruit firmness

Chlorophyll and total anthocyanins were extracted and assayed essentially as described before (Hiscox and Israelstam, 1979; Given *et al.*, 1988b). Fruit texture measurements were made using a penetrometer (Woolley *et al.*, 2001) and firmness was assessed as the maximum force (g) recorded at the yield point as the probe travelled 5mm into the fruit.

Isolation of nucleic acids

Total nucleic acids were isolated from fruit and leaf tissues based on a modified CTAB method (Chang *et al.*, 1993). Total nucleic acids were precipitated from the CTAB extract with isopropanol and further purified with SSTE buffer before separating total RNA from DNA with 2M LiCl.

5' and 3' RACE of full-length FaMADS4 cDNA

Total RNA isolated from cultivar Calypso ripe fruit was the template for 5' and 3' RACE using the GeneRacer Kit (Invitrogen, Paisley, UK) essentially according to the manufacturer's protocol. For the reverse transcription step, Superscript II reverse transcriptase (Invitrogen) was used with the anchored RT primer: 5'-GCGAGCACAGAATTAATACGACTCACTATAGG(T)₁₂VN-3'. Gene-specific forward and reverse primers were designed from the predicted *F. nubicola* cDNA sequence and nested PCRs performed with KOD Hot Start polymerase (Invitrogen) together with GeneRacer adapter primers. Amplicons were either sequenced directly or cloned into the pJET1.2 vector (Fermentas, York, UK) for sequencing.

Gene expression assays for FaMADS9

Expression of the strawberry *FaMADS9* gene in receptacle tissue was determined by absolute quantification using a two-step real-time PCR method. Total RNA from each tissue was initially reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol but without added dithiothreitol. Synthesis of first strand cDNA was primed with the gene-specific primer: 5'-ATCCTTAGTAAACCAGTCTTT-3'. Gene expression was analysed by real-time PCR using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). The PCR reaction (10µl) contained 5µl qPCR Mastermix Plus for SYBR Green (Eurogentec, Southampton, UK) with a passive reference dye, first strand cDNA template synthesized from 20ng total RNA, and 200nM of each primer. Absolute standards were prepared from a pGEMT (Promega, Southampton, UK) plasmid containing the *FaMADS9* cDNA. Following an initial denaturation of 95°C for 1min 30s, the thermal cycling programme was 40 cycles of 95°C for 30s, annealing at 60 °C for 30s, and extension at 72 °C for 30s. Three technical replicates were run for each of the standards and the unknowns and three fruits were sampled at each stage from each line. The C_t value for each QRT-PCR was determined and a standard curve used to calculate absolute amounts of target cDNA. Results were expressed as the number of transcript copies per microgram total RNA. Forward and reverse primers for the strawberry *FaMADS9*-gene were *FaMADS9* QRT-PCR F3: 5'-AGCCAACAGAGATTGAAAACGAAG-3' and *FaMADS9* QRT-PCR R3: 5'-GACACCACAGCATTGTACCCTATTTG-3'. The primers span an intron and prevent any detectable amplification from genomic DNA (up to 50ng). For sensitive detection of strawberry *FaMADS9* expression in petals and achenes a nested real-time PCR method was used. The primary PCR contained 200nM of the forward and reverse primers *FaMADS9* QRT-PCR F3 and *FaMADS9* QRT-PCR R3 in a 20µl reaction containing 10µl Megamix Blue Double PCR mixture (Microzone, HaywardsHeath, UK) and 20ng first strand cDNA. The reaction was initially denatured at 95 °C for 1min 30s, followed by thermal cycling for 15 cycles of 95 °C for 30s, annealing at 60 °C for 30s, and extension at 72 °C for 30s. Template from the primary reaction (1µl) was used in the secondary real-time PCR containing the nested primers *FaMADS9* QRT-PCR F4: 5'-CAGAGATTTGAAAACGAAGTTGGATG-3' and *FaMADS9* QRT-PCR R4: 5'-TGTTGAGTTCATATAGCATAGTCTGGTG-3'.

Gene expression assays for FaMADS4

To quantify the low expression of *FaMADS4* in fruit tissue, a nested real-time PCR method similar to that for *FaMADS9* was used. The primary PCR contained 200nM of the forward and reverse primers *FaMADS4* QRT-PCR F1:5'-CTACAACGCACG-CAGAGACG-3' and *FaMADS4* QRT-PCR R1:5'-GTCGCATTGTAAACGCTCAA-3' in a 10µl reaction containing 5µl Megamix Blue Double PCR mixture (Microzone) and 10ng first strand cDNA. Each primer spanned an intron-exon border.

The reaction was initially denatured at 95 °C for 1min 50s, followed by thermal cycling for 15 cycles of 95 °C for 10s, annealing at 55 °C for 30s, and extension at 72 °C for 20s. Template from the primary reaction (0.5µl) was used in the secondary real-time PCR containing 200nM of the inner (nested) primers *FaMADS4* QRT-PCR F2: 5'-AGCTTCAGCAGCTTGAGAAT-3' and *FaMADS4* QRT-PCR R2: 5'-CTTCATTACTCTTCCAACCTCA-3'. This reaction (10µl) contained 5µl Mesa Green qPCR Mastermix Plus for SYBR assay (Eurogentec) with passive reference dye. Absolute standards were prepared from sequenced purified products of a PCR with *FaMADS4* QRT-PCR F1/R1 primers. Following an initial activation of 95°C for 5min, the thermal cycling programme was 40 cycles of 95 °C for 15s, annealing at 55 °C for 20s, and extension at 72 °C for 40s.

Gene expression assays for all other genes

A two-step real-time PCR method similar to that for *FaMADS9* was used for determining the expression of other genes in receptacle tissue, except that first strand cDNA synthesis, primed with oligo dT₁₂₋₁₈, cDNA template from 10ng of RNA, was used and absolute standards were purified RT-PCR products. The following genes (GenBank accession numbers in parentheses) were assayed: *AGL6-like* MADS-box gene (DV440581), *SEP3-like* MADS-box gene (DV440160), *SHATTERPROOF2-like* MADS-box gene (CO380891), *AGAMOUS-like* MADS-box gene (AF168468), endo-1,4-β-D-glucanase (AF041405), chalcone synthase (AY997297), flavanone-3-hydroxylase (AY691919), and quinoneoxidoreductase (AY158836). Primers are described in Supplementary Table S3 at *JXB* online.

Cross-species arrays

Microarray experiments were conducted to quantify transcriptional differences between fruit from two transgenic strawberry lines (MADS A and MADS I) and wild-type plants. Since no large-scale strawberry microarrays are currently available, a cross-species experiment was performed using the Affymetrix *Arabidopsis thaliana* ATH1-121051 GeneChip® array (ATH1 GeneChip®; Hammond *et al.*, 2005, 2006). Briefly, genomic DNA isolated from strawberry was biotinylated and hybridized to an ATH1 GeneChip® array as described previously (Hammond *et al.*, 2005). The ATH1 GeneChip® array was then scanned and a gDNA hybridization cell intensity file generated (.CEL). Probe-pairs from the gDNA CEL file were selected for subsequent transcriptome analysis of the strawberry fruits using a parser written in Perl (www.perl.com; Hammond *et al.*, 2005) to generate custom probe mask files. The Perl script selected probe-pairs in which the PM probe has agDNA hybridization intensity greater than a user-defined threshold, and hence at the defined intensity, probe-pairs with a lower value are defined as having poor hybridization between the strawberry gDNA and the *Arabidopsis* ATH1 GeneChip probe and would not be representative of the gene. These probes are not useful for transcriptional analyses. A gDNA-based probe-selection at a hybridization intensity threshold of 250 was selected for analysis of transcriptional data. This threshold was selected as optimal, based on (i) the retention of 81% of probe-sets with over 84% represented by two or more probe-pairs (see Supplementary Fig. S1A at *JXB* online) and (ii) the maximal detection of significantly ($P < 0.05$) differentially expressed genes between the MADS A and MADS I transgenic lines and wild-type (see Supplementary Fig. S1B at *JXB* online).

Total RNA was extracted from red ripe receptacle tissue (minus achenes) of MADS A, MADS I, and wild-type plants as described above. Three replicates, consisting of tissue from single fruits were performed. Total RNA was labelled and hybridized to Affymetrix-*Arabidopsis thaliana* GeneChip arrays using standard protocols (Affymetrix, Santa Clara, CA, USA). GeneChip arrays were scanned using an Affymetrix 3000 GeneArray scanner and cell intensity files (.cel files) were generated. The RNA.cel files were

loaded into GeneSpring GX (Agilent Technologies, Palo Alto, CA, USA) using the RMA normalization algorithm and the probe mask file generated from the gDNA hybridization (Hammond *et al.*, 2005). Initially, genes significantly ($P < 0.05$) differentially expressed between either of the transgenic lines and the wildtype by more than 1.3-fold were identified using a one-way ANOVA with a Benjamini and Hochberg False Discovery Rate of 5% to correct for multiple testing (see Supplementary Table S1 at *JXB* online). Hierarchical clustering was performed using a Pearson correlation similarity measure in GeneSpring GX (Agilent Technologies). Common genes significantly differentially expressed between both *FaMADS9* transgenic lines and the wild-type were identified by combining the two data sets and performing the one-way ANOVA as above (see Supplementary Table S2 at *JXB* online).

Results

Phylogenetic analysis of strawberry *FaMADS9*

The relationship between *FaMADS9*, isolated by Vrebalov *et al.* (2002), and other SEP proteins in selected fleshy fruit species as compared with the SEP1/SEP2/SEP3/SEP4 clade of *Arabidopsis*, a dry-fruited species, was analysed by constructing a phylogenetic tree (Fig. 1). The phylogram suggests that *FaMADS9* belongs to the SEP1/2 clade. By contrast, the tomato *LeMADS-RIN* is more closely aligned with SEP4. Strawberry belongs to the Rosaceae family and *FaMADS9* is most similar to the SEP1/2 proteins *MdMADS1*, 8, and 9 and *PpMADS7* in the related apple (*Malus X domestica* Borkh.) and peach (*Prunus persica*) species, respectively. A new SEP gene (*FaMADS4*) has also been isolated from strawberry (described below and included in Fig. 1) encoding a protein that is more closely related to SEP4 and *LeMADS-RIN* than *FaMADS9*. However, this gene was expressed at a very low level in ripening fruit and was not investigated further. A SEP4-like homologue, *MdMADS4*, is also present in apple.

Down-regulation of a fruit-related SEP1/2 gene in strawberry alters normal fruit development and ripening

To examine the function of *FaMADS9*, transgenic strawberry plants were produced containing a 552 bp fragment of the *FaMADS9* gene sequence in the antisense orientation ectopically expressed using the cauliflower mosaic virus (CaMV) 35S promoter. Ten independent T₀ plants were generated in tissue culture and three lines gave plants with markedly altered flower and fruit phenotypes. The primary transformants were vegetatively propagated from runners (stolons) and these clones were then used for further analysis.

Clones from all three transgenic lines (A, I, and J) produced flowers with petals that exhibited increasing green colouration in the order A < I < J compared with the normally white petals of the untransformed plants (Fig. 2A). In normal plants, the petals abscise 2–3d after anthesis. Abscission of petals in the transgenic plants was either delayed (A) or was totally abolished (I and J). Line J exhibited such a severe developmental phenotype that the fruit failed to advance beyond the immature stage and were not analysed further. In lines A and I, it was observed that

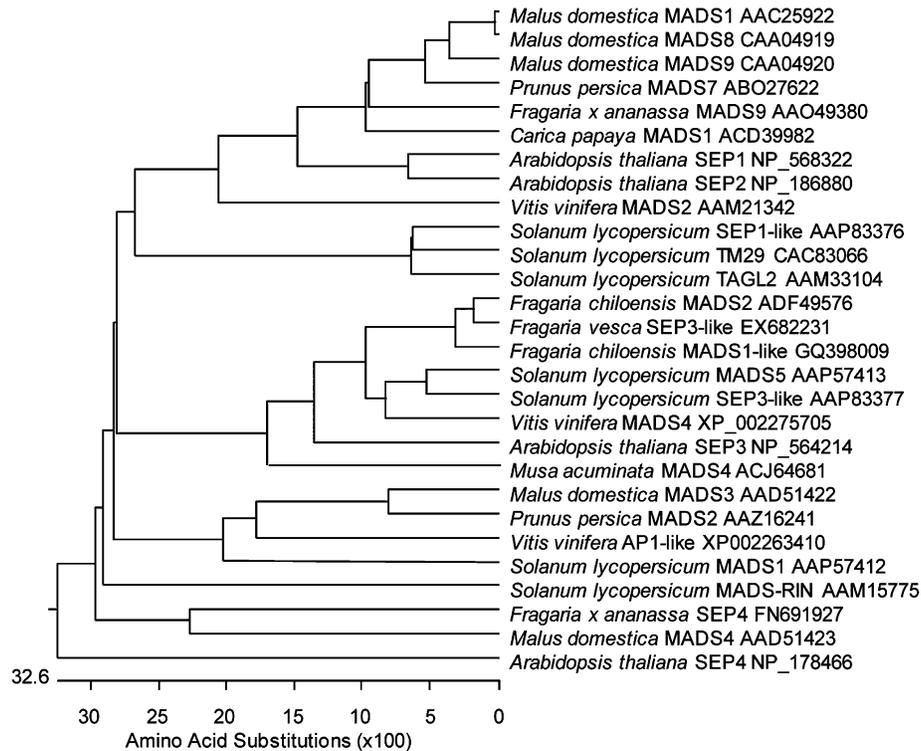


Fig. 1. Phylogenetic analysis of SEPALLATA proteins in selected fruit species. A phylogenetic tree of full-length protein sequences from apple (*Malus domestica*), *Arabidopsis*, banana (*Musa acuminata*), grape (*Vitis vinifera*), papaya (*Carica papaya*), peach (*Prunus persica*), strawberry (*Fragaria x ananassa*), and tomato (*Solanum lycopersicum*) was constructed using MegAlign software (DNASTar) after aligning by ClustalW.

self-pollination occurred much less readily than in the wild-type controls and hand pollination was necessary for optimum fruit expansion. This was consistent with altered anther development and pollen release observed in the flowers from these plants (Fig.2B). The antisense lines A and I achieved normal fruit expansion with no significant differences in morphology prior to ripening, but showed altered ripening in both receptacle and achenes. The fruit of the strawberry is a false fruit, the fleshy part being derived from the receptacle while the true fruits ‘achenes’ are embedded in the surface of the receptacle. The onset of ripening, observed as an increase in the production of red anthocyanin pigments in the receptacle, was delayed by around 10d compared with wild-type plants. Ripe fruits sampled from the transgenic lines were on average 11d (A) and 12d (I) older than the wild-type control to achieve comparable developmental stages. Unlike wild-type fruits, where ripening is normally initiated throughout the receptacle, anthocyanin accumulation in the transgenic lines was often observed first at the distal end before it progressed towards the calyx (Fig.2A). Chlorophyll was retained in the achenes of line I, which remained green with indications of a similar trend in A (Fig.3A). By contrast, there were no differences in chlorophyll content in receptacle tissue between the transgenic and wild-type receptacles (data not shown). Examination by light microscopy did not reveal any significant structural differences in the achenes from these transgenic lines. Although anthocyanin

production increased in the developing receptacles of line A and I, it never attained the levels found in wild-type fruits (Fig.3B). Anthocyanin content in the achenes was not measured due to insufficient tissue for reliable determination. The fruits from lines A and I retained a significantly firmer texture than wild-type controls, as determined by measuring tissue yield force at the stage where the fruits were red ripe and ready for consumption (Fig.3C).

Comparison of *FaMADS9* target gene expression in receptacles, achenes, and petals of wild-type and antisense lines

In order to determine low levels of *FaMADS9* expression in achene and petal tissue a modified quantitative RT-PCR (QRT-PCR) method for single copy detection was used. In wild-type plants *FaMADS9* was expressed in developing receptacles, achenes, and petals (Fig.4A–C). The fruit and petal phenotypes of the antisense lines described above were associated with a significant reduction in *FaMADS9* expression in these tissues (Fig.4A–C). The petals showed a significant reduction in the level of expression of the gene even though it was an order of magnitude lower in this tissue than in achenes or receptacles (Fig.4C). Furthermore, line I, which had the more severe petal and fruit phenotypes, almost always displayed the lowest levels of *FaMADS9* expression (Fig.4A–C).

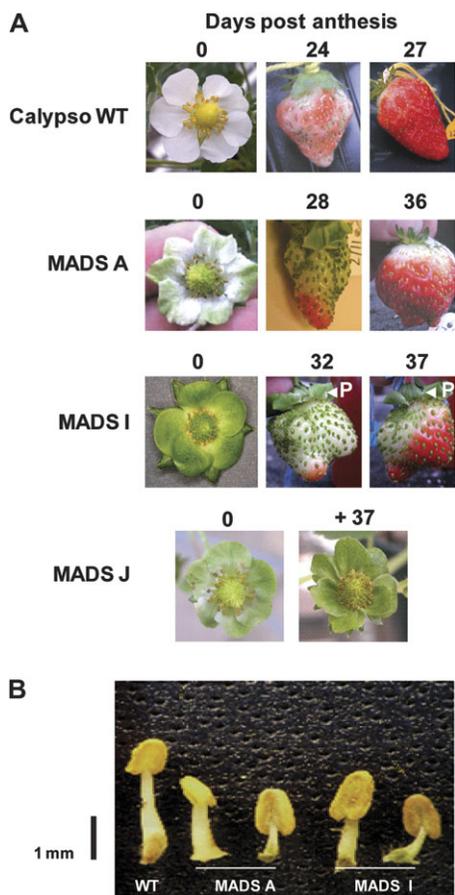


Fig. 2. A MADS-box gene affects flower and fruit development in strawberry. (A) Altered development of flowers and fruit in untransformed (WT) strawberry and three independent lines (MADS A, MADS I, and MADS J) transformed with an antisense strawberry *FaMADS9* transgene sequence. P, attached petals. (B) Anthers in two independent transformed lines (MADS A and MADS I).

Effect of repressing *FaMADS9* on the programme of ripening-related gene expression in strawberry

To assess downstream gene expression effects in the *FaMADS9* transgenic lines, cross-species microarray experiments were performed. The Affymetrix *Arabidopsis thaliana* ATH1-121051 GeneChip® array was used, since comprehensive public strawberry array resources were not available. Although *FaMADS9* cDNA was isolated using a tomato probe and tomato oligo microarrays are available, the choice was made to use the array from *Arabidopsis* because this species is phylogenetically more closely related to strawberry than tomato (Judd *et al.*, 1999) and this array is more comprehensive than that of tomato. A mixed physical and bioinformatic method was used to optimize the analysis of the transcriptional data (Hammond *et al.*, 2005, 2006). Briefly, genomic DNA (gDNA) from strawberry was hybridized onto the ATH1 GeneChip array and a parser script, written in Perl, was used to remove probe-pairs whose perfect match (PM) probe signal intensity value was below a user-defined gDNA hybridization intensity thresh-

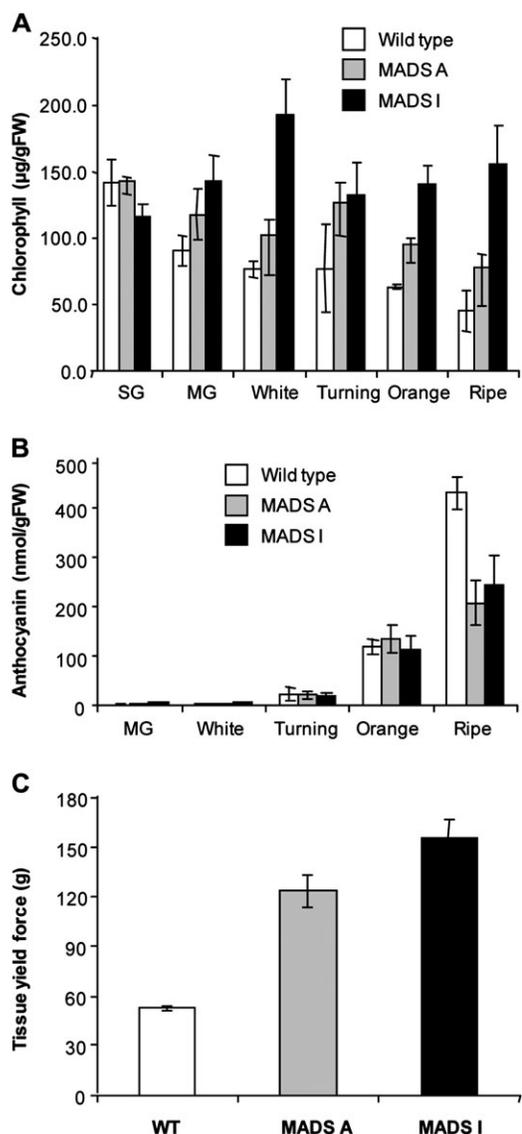


Fig. 3. Repression of strawberry *FaMADS9* alters normal ripening. (A) Chlorophyll content in achenes; (B) anthocyanin content in receptacle tissue of three independent fruits sampled at each stage of development; (C) firmness of receptacle tissue. Measurements were made on 43, 35, and 30 ripe fruits from untransformed, MADS A and MADS I plants, respectively. Bars represent mean values (\pm SEM) of untransformed wild type (white) and independent antisense transformants MADS A (grey) and MADS I (black). SG and MG are small green and mature green fruits, respectively.

old. The optimum gDNA hybridization intensity threshold was selected based on the retention of probe-sets and maximal detection of differentially expressed transcripts (see Supplementary Fig.S1 at *JXB* online). Thus, only probes with good homology (gDNA hybridization signal intensity ≥ 250) to strawberry gDNA were used to analyse the transcriptional data.

Hierarchical clustering of genes significantly ($P < 0.05$) differentially expressed in red ripe receptacle tissue between

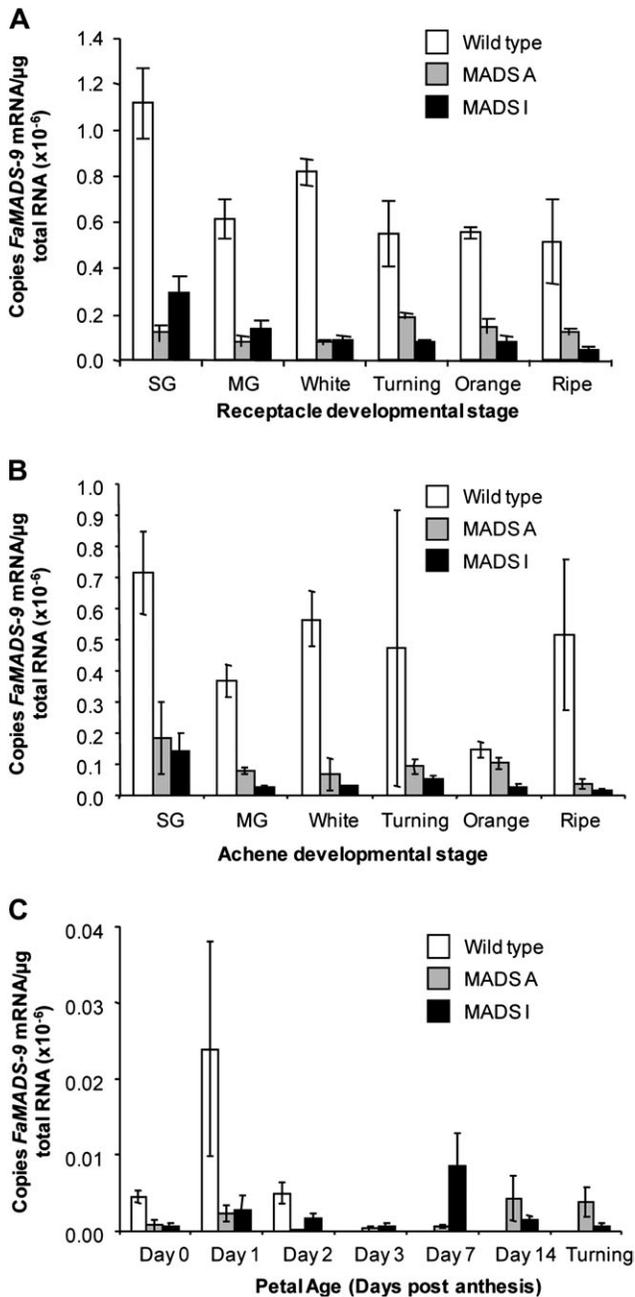


Fig. 4. *FaMADS9* gene expression in strawberry receptacles, achenes and petals. (A) Receptacle tissue; (B) achenes; (C) petals. Expression of an endogenous *RIN*-like gene was determined by absolute QRT-PCR. Bars indicate the mean values (\pm SEM) of three fruits sampled from each line (see Fig. 2) at different stages of development. Data for wild-type petals only extends to day 2 as from this point these petals abscised.

the lines A and I and the wild-type, identified groups of genes with similar expression profiles (see Supplementary Fig.S2 and Supplementary Table S1 at *JXB* online). Down-regulated transcripts in clusters I and II included those with homology to *Arabidopsis AGL* (*AGL5*; At2g42830), MYB (At5g12870 and At5g55020), F-box (At1g64290, At2g17020, and At3g06380), and bHLH (At4g36540) transcription factor families. Also present in these clusters were

transcripts with homology to *Arabidopsis* cell wall-related genes (pectin methylesterase inhibitor, At5g62360; cellulase, At1g70710; pectate lyase, At4g13710; and pectinesterase, At5g49180) and an ACC oxidase (At2g19590) gene. From the Gene Ontology (GO) annotations associated with the homologous *Arabidopsis* transcripts, several groups of genes were identified that were significantly ($P < 0.05$) over-represented in Clusters I and II, including those involved in the ubiquitin cycle (GO:6512), ovule and carpel development (GO:48481 and GO:48440), and signal transduction (GO:7165). Transcripts with elevated levels in both transgenic lines (cluster V) had homology to members of the *Arabidopsis* F-box (At2g15640), bZIP (At1g68640), and MYB (At1g72650 and At2g01060) transcription factor families, as well as two *AGL* (*AGL16*; At3g57230 and *AGL24*; At4g24540) and two *NAM* genes (At1g12260 and At3g04060). Genes up-regulated in A or I lines (clusters III and IV, respectively) included those with homology to *Arabidopsis CLAVATA1* (At1g75820) and pectinesterase 2 (At1g53830).

To distinguish genes differentially expressed in both *FaMADS9* transgenic lines, the data sets from each were combined and compared with the wild-type. In total, 87 genes were identified, 38 with higher and 49 with lower transcript abundance in the transgenic lines compared with the wild-type (see Supplementary Table S2 at *JXB* online). These included transcripts with homology to *Arabidopsis AGL5*, three F-box genes (At1g64290, At2g15640, and At2g17020), an endo-1,4- β -D-glucanase (At1g70710), and a homeobox gene (At1g05230).

Based on the microarray analyses, a number of genes whose expression was down-regulated in the *FaMADS9*-silenced lines were selected for further expression analysis by QRT-PCR (Fig.5). Strawberry genes whose sequences were available in public databases were identified from the corresponding *Arabidopsis* probes. Three genes that were identified as being down-regulated in both of the transgenic lines MADS A and MADS I were a *SEP3-like* MADS-box gene (GenBank DV440160; Fig.5A), a *SHATTERPROOF2-like* MADS-box gene (GenBank CO380891; Fig.5B), and an endo-1,4- β -D-glucanase (GenBank AF041405; Fig.5C). The QRT-PCR analyses confirmed that all these genes were repressed in the red ripe transgenic fruit. The expression of two other genes related to *FaMADS9*, an *AGL6-like* MADS-box gene (GenBank DV440581) and an *AGAMOUS-like* MADS-box gene (GenBank AF168468) were also investigated. The *AGL6-like* MADS-box gene was repressed in red ripe receptacle tissue of both transgenic lines in the order MADS I > MADS A > WT whereas expression of the *AGAMOUS-like* MADS-box gene did not show a trend related to the phenotypic differences of these plants (Fig. 5E,F, respectively). In addition, the expression of two genes related to anthocyanin biosynthesis, chalcone synthase and flavanone-3-hydroxylase, and a gene involved in flavour biogenesis, quinoneoxidoreductase (GenBank accessions AY997297, AY691919, and AY158836, respectively) were also assayed by QRT-PCR. Although the expression of these genes is known to be enhanced during the ripening of normal fruit,

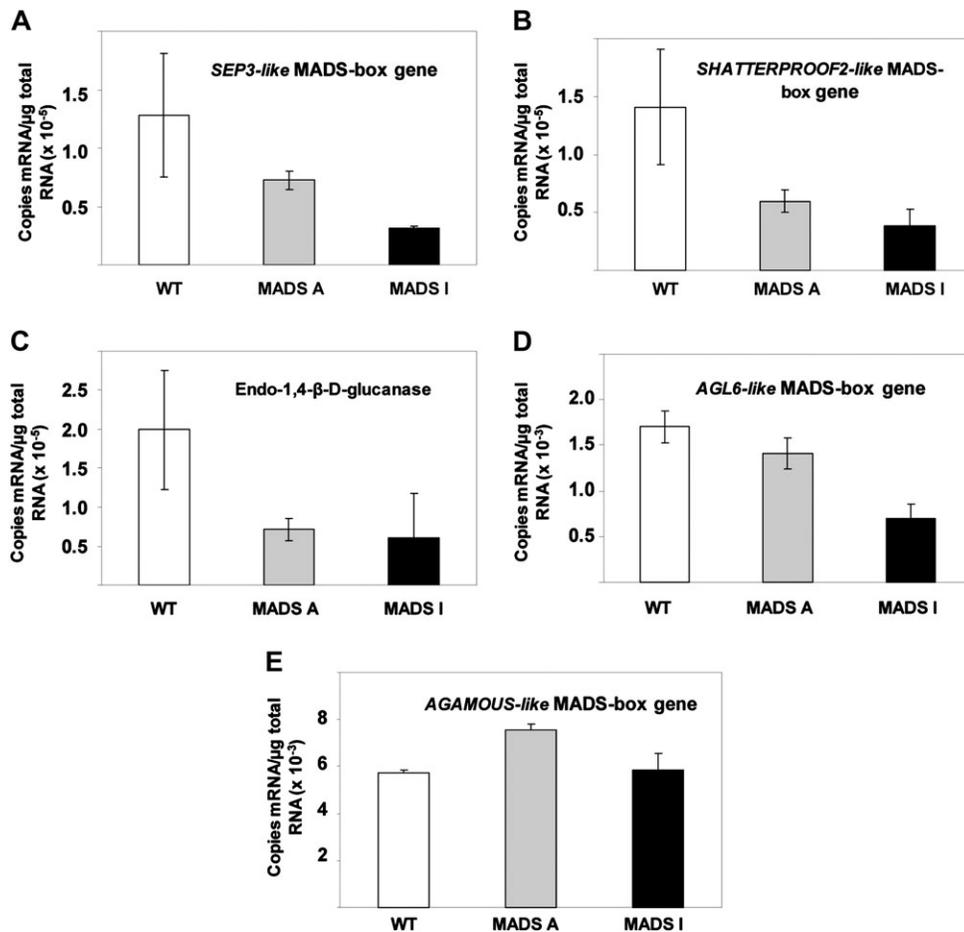


Fig. 5. Expression of selected strawberry genes determined by absolute QRT-PCR. (A) *SEP3-like* MADS-box gene (DV440160); (B) *SHATTERPROOF2-like* MADS-box gene (CO380891); (C) endo-1,4-β-D-glucanase (AF041405); (D) *AGL6-like* MADS-box gene (DV440581); (E) *AGAMOUS-like* MADS-box gene (AF168468). Bars represent mean values (\pm SEM) of three fruits sampled from each line (see Fig. 2).

they were not identified from microarray data as having altered expression in the transgenic lines, and this was confirmed by QRT-PCR (see Supplementary Fig.S3 at *JXB* online).

Cloning and expression analysis of *FaMADS4*

The sequence of a putative orthologue of *SEP4* has been retrieved from a WGS sequence (kindly provided by DJ Sargent, unpublished data) of *Fragaria nubicola* cultivar '601', a diploid species of strawberry, using tBlastN. From this information primers were designed to clone a full-length 985 bp cDNA from the octaploid *Fragaria ananassa* (GenBank accession FN691927). This cDNA encodes a protein with 51% identity over 268 amino acids to *LeMADSRIN*, and which amongst *Arabidopsis* MADS-box proteins has the highest similarity to the *SEP4* transcription factor (45% identity over 280 amino acids). The corresponding gene has been named *FaMADS4*.

In WT fruit the *FaMADS4* gene is expressed at levels <1% of those of *FaMADS9* and shows a modest increase in expression from the mature green to the orange stage

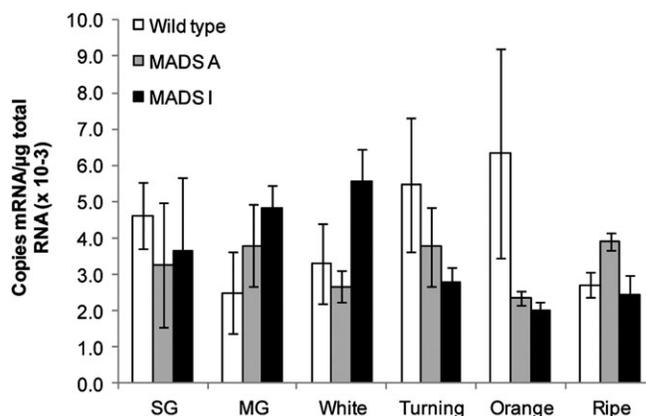


Fig. 6. Expression of *FaMADS4* in strawberry receptacle. Expression of a *SEP4-like* gene was determined by absolute QRT-PCR. Bars indicate the mean values (\pm SEM) of three fruits sampled from each line (see Fig. 2) at different stages of development.

(Fig.6). Expression of *FaMADS4* was not substantially altered in the transgenic fruits down-regulated by the *FaMADS9* construct, although the expression was reduced at the orange stage.

Discussion

Fleshy fruits have traditionally been placed in one of two classes dependent on their ripening behaviour. In climacteric fruits, such as tomato and banana, ripening is associated with a burst of respiration and is initiated and co-ordinated by ethylene. By contrast, ripening in non-climacteric fruits such as strawberry and grape appears to be an ethylene-independent process (Giovannoni, 2004). Fruit in both classes, however, show changes in colour, texture, and flavour that probably reflect similar programmes of gene expression (Fei *et al.*, 2004).

MADS-box transcription factors are expressed in a range of species with fleshy fruits including tomato, banana, apple, grape, and strawberry (Vrebalov *et al.*, 2002; Fei *et al.*, 2004; Malcomber and Kellogg, 2005). In tomato it has been shown that a *SEP4-like* gene (*LeMADS-RIN*) is necessary for ripening (Vrebalov *et al.*, 2002). At the N-terminal region SEP proteins have highly conserved MADS-box/MEF2 and K-box domains involved in transcription factor binding and protein–protein interactions, respectively. The C-terminal region, however, is highly variable and confers important functional properties on the protein. This region in *LeMADS-RIN* is essential for normal fruit development in tomato and its disruption accounts for ripening inhibition in the *rin* mutant (Ito *et al.*, 2008). It has been demonstrated here that a gene of the *SEPI12* subfamily can modulate the development and ripening of strawberry receptacle, achene, and petal tissues. Like the *SEP4 LeMADS-RIN* gene in tomato, suppression of the strawberry *SEPI12 FaMADS9* gene delays normal ripening. By contrast, silencing of *TM29*, a *SEPI12-like* gene, in tomato produced parthenocarpic fruit and was not reported to affect ripening (Ampomah-Dwamena *et al.*, 2002). Genes of the *SEP3* clade are relatively highly expressed in banana fruit, but functional studies showed they were not able to complement ripening in the tomato *rin* mutant (Elitzur *et al.*, 2010). These observations indicate that different classes of *SEP* genes can have non-redundant and specialized functions in fleshy fruit species.

In the *rin* tomato mutant, where the functional MADS-box transcript is abolished, the fruit show complete inhibition of ripening in the homozygous state, but in heterozygous individuals delayed ripening is apparent, presumably due to the presence of lower than normal levels of *LeMADS-RIN* transcript. This latter situation is probably analogous to our antisense transgenic strawberry fruit exhibiting moderate levels of *FaMADS9* expression. However, in contrast to tomato where a *SEP4-like* gene is involved in the control of ripening, severe repression of the *FaMADS9 SEPI12-like* gene in strawberry results in a complete inhibition of receptacle development. The expression pattern of *FaMADS9* is consistent with its involvement in both fruit development and ripening.

We wanted to investigate further if a ripening-related homologue of the tomato *SEP4 LeMADS-RIN* gene could be found in strawberry. No *SEP4* genes have been identified from strawberry fruit EST libraries in the public databases. However, recent information from high throughput genomic

sequencing of *Fragaria nubicola*, a diploid species, has allowed us to clone a *SEP4-like* gene (*FaMADS4*) from *Fragaria ananassa* (cv. Calypso), an octoploid species. However, expression analysis revealed very low levels of transcript in ripening fruits, and no consistent relationship between mRNA levels of the strawberry *SEP4* and inhibition of fruit development or ripening in the transgenic lines. The relatively low expression of the *FaMADS4* gene may explain why *FaMADS9* was the only cDNA isolated from a strawberry ripe fruit cDNA library using the tomato *LeMADS-RIN* probe, and also why our microarray data did not show any evidence of differential *SEP4* expression between the wild type and transgenic lines.

The possibility was considered that the antisense configuration of the *FaMADS9* transgene might be acting by suppressing both its own transcription and that of certain other related MADS-box genes. However, analysis of strawberry ESTs and changes in the expression of related gene sequences in our transgenic plants suggested that this was not the case. A BlastN search of approximately 21000 strawberry nucleotide sequences available in GenBank found four MADS-box gene sequences with some similarity to the 552 bp *FaMADS9* transgene. These were *SEP3-like* (83% identity over 213 bp), *AGL6-like* (84% identity over 172 bp), *AGAMOUS-like* (89% identity over 68 bp), and *SHATTER-PROOF2-like* (87% over 47 bp). The microarray data, later confirmed by QRT-PCR, identified that the *SEP3-like* and *SHATTERPROOF2-like* genes were down-regulated in the transgenic lines. The sensitive QRT-PCR method also detected suppression of an *AGL6-like* MADS-box gene in strawberry receptacles. However, repression of these genes was much less pronounced than that of *FaMADS9* in this tissue. These data are entirely consistent with the remarkable specificity shown by antisense transgenes (Palecanda and Sharrock, 2001). These observations strongly suggest that the suppression of this gene alone is responsible for altering the development and ripening of strawberry fruit.

Retention of chlorophyll in the achenes of our antisense A and I lines is suggestive of the maintenance of a vegetative state and the arrest of ripening in these organs. Achenes are the true fruits of the strawberry and play a fundamental role in the growth and development of the receptacle through the action of auxin (Nitsch, 1950; Given *et al.*, 1988a). The achenes resemble dry fruits and also undergo ripening changes during normal development including degradation of chlorophyll, accumulation of anthocyanins, and loss of water. The development and ripening of the receptacle is known to be influenced by the achenes (Given *et al.*, 1988a). *FaMADS9* may co-ordinate these events in both tissues.

The antisense transgene also altered petal development. The ‘green petal’ phenotype of *FaMADS9* lines resembled the effects of silencing the *SEPI12-like* gene *TM29* in tomato (Ampomah-Dwamena *et al.*, 2002). This contrasts with the lack of an observable petal phenotype in tomato when the *SEP4 LeMADS-RIN* was suppressed. This tomato gene is mainly expressed in fruit tissue, but like *FaMADS9* is also expressed in the inflorescence (Malcomber and Kellogg, 2005). Similarly, the lack of an observable phenotype in

other tissues does not exclude the possibility that these genes might be expressed in other parts of the plant. The *SEP* genes in *Arabidopsis* show considerable functional redundancy in controlling the identity of floral organs. Our findings, together with those reported for tomato, reinforce the view that *SEP* genes play a major role in the developmental regulation of ripening; however, they also suggest the specific role of each class of *SEP* gene may vary according to tissue and species (Malcomber and Kellogg, 2005; Zahn *et al.*, 2005).

To study gene expression profiles in strawberry, as influenced by the antisense *FaMADS9* transgene, an *Arabidopsis* microarray was used. Cross-species array experiments have now been demonstrated to provide a useful measure of the transcriptome where species-specific arrays are inadequate or not publicly available (Hammond *et al.*, 2005). Nevertheless, while cross-species hybridizations can be optimized for utility via incorporation of appropriate controls (such as genomic DNA hybridization as described in this study) the results of the cross-species arrays should be interpreted with caution. For example, every hybridization reaction cannot be independently verified and the evolutionary distance between the species may result in cross-hybridization between members of the same gene family. However, the validation of a subset of these genes by QRT-PCR has shown this approach to be robust for the analysis of the strawberry transcriptome, even with TFs that may be expected to be transcribed at relatively low levels. Using this approach, it has been possible to investigate the expression of a wide range of genes in strawberry without any pre-selection based on limited information available in the literature and to identify novel gene expression associated with ripening and changes caused by altered *FaMADS9* expression.

Our microarray experiments have revealed the pleiotropic effects of silencing the *FaMADS9* transcript, including effects on the expression of both regulatory and metabolic genes. SEP proteins are known to form multimers (Malcomber and Kellogg, 2005) and it is likely that these complexes are necessary for ripening. Several MADS-box transcription factors, including those with sequence homology to *Arabidopsis SEP3* and *SHP 2* were among the genes repressed in the transgenic strawberry fruits. Interestingly, the *SHP* genes are involved in silique development and dehiscence in the dry fruits of *Arabidopsis*. Although there is no analogous process to dehiscence in strawberry fruits, both fruit types undergo cell separation events leading either to the parting of tissues in the dehiscence zones of siliques or softening in the fleshy receptacle. These cell wall degradation processes may be controlled directly or indirectly by this class of MADS-box genes (Ferrández, 2002). In the current study, the transgenic fruits showed reduced pectate lyase and endo-1,4- β -D-glucanase expression consistent with enhanced fruit firmness. Tomato also shows induction of an *SHP-like* gene (*TAGL1*) during development and ripening and, recently, *TAGL1* has been shown to be involved in tomato fruit development and ripening (Vrebalov *et al.*, 2009). By contrast, neither *SEP3-like* nor *SEPI12-like* genes appear to have a clear fruit-related

function in tomato (Ampomah-Dwamena *et al.*, 2002; Fei *et al.*, 2004). The induction of a likely *SEP3* orthologue, but not *MADS-RIN* or *FaMADS9* homologues, has been observed in ripening grapes, another non-climacteric fruit (Fei *et al.*, 2004).

Differences may be expected between the regulatory factors involved in controlling ripening in fruiting species given the diversity of fruit forms in the Angiosperms. However, our work provides evidence that MADS-box genes are common features of the ripening regulatory network in both climacteric and non-climacteric fruits. The challenge will be to determine how these master switches are modulated by hormones such as auxin and ethylene, in addition to their links to the downstream effectors bringing about changes in fruit colour, texture, and flavour.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Validation of gDNA test for microarray.

Supplementary Fig. S2. Gene expression profiles in wild-type and *FaMADS9* transgenic strawberry fruit.

Supplementary Fig. S3. Expression of chalcone synthase, flavanone-3-hydroxylase, and quinoneoxidoreductase in strawberry receptacles determined by absolute QRT-PCR.

Supplementary Table S1. Normalized probe-set signal values of genes significantly ($P < 0.05$) differentially (> 1.3 -fold) expressed in *FaMADS9* transgenic fruits compared with wild-type fruits.

Supplementary Table S2. Normalized probe-set signal values of genes significantly ($P < 0.05$) differentially (> 1.3 -fold) expressed in both *FaMADS9* transgenic fruits compared with wild-type fruits.

Supplementary Table S3. Primer sequences for QRT-PCR of strawberry genes other than *FaMADS9*.

Acknowledgements

We would like to thank the Biotechnology and Biological Sciences Research Council (UK) and the Department for Environment, Food and Rural Affairs (UK) for financial support. Also Malcolm Bennett (University of Nottingham) for useful discussions during the preparation of this manuscript.

Conflict of interest statement: No conflicts declared.

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