The MADS-box gene FveSEP3 plays essential roles in flower organogenesis and fruit development in woodland strawberry

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Abstract
Flower and fruit development are two key steps for plant reproduction. The ABCE model for flower development has been well established in model plant species; however, the functions of ABCE genes in fruit crops are less understood. In this work, we identified an EMS mutant named R27 in woodland strawberry (Fragaria vesca), showing the conversion of petals, stamens, and carpels to sepaloid organs in a semidominant inheritance fashion. Mapping by sequencing revealed that the class E gene homolog FveSEP3 (FvH4_4g23530) possessed the causative mutation in R27 due to a G to E amino acid change in the conserved MADS domain. Additional fvesep3CR mutants generated by CRISPR/Cas9 displayed similar phenotypes to fvesep3-R27. Overexpressing wild-type or mutated FveSEP3 in Arabidopsis suggested that the mutation in R27 might cause a dominant-negative effect. Further analyses indicated that FveSEP3 physically interacted with each of the ABCE proteins in strawberry. Moreover, both R27 and fvesep3CR mutants exhibited parthenocarpic fruit growth and delayed fruit ripening. Transcriptome analysis revealed that both common and specific differentially expressed genes were identified in young fruit at 6–7 days post anthesis (DPA) of fvesep3 and pollinated wild type when compared to unpollinated wild type, especially those in the auxin pathway, a key hormone regulating fruit set in strawberry. Together, we provided compelling evidence that FveSEP3 plays predominant E functions compared to other E gene homologs in flower development and that FveSEP3 represses fruit growth in the absence of pollination and promotes fruit ripening in strawberry.

Introduction
The octoploid strawberry species Fragaria × ananassa is cultivated worldwide as an economically important fruit crop. The diploid woodland strawberry Fragaria vesca has emerged as a model species for research on flower development and fruit ripening1,2. The flower is an essential organ in angiosperms for fruit production. F. vesca flowers have four typical whorls of floral organs, namely, sepals, petals, stamens, and carpels. A special feature of strawberry flowers is that numerous carpels are independently developed and attached to the receptacle, which is enlarged to generate juicy flesh, while dry achenes are botanical true fruits3. Given the importance of flowers, the developmental control of strawberry flowers remains unclear.

To date, the well-known ABCE model for flower development explains the basic tenets of floral organ specification. In this model, four classes of homeotic genes (A, B, C, and E) work coordinately to determine the identity of each whorl of floral organs. Specifically, class A genes (APETALA1, AP1; APETALA2, AP2) specify sepal identity; class A and B (APETALA3, AP3, PISTILLATA, PI) genes specify petal identity; class B and C (AGAMOUS, AG) genes specify stamen identity; class C genes...
specify carpel identity; and class E genes (SEPALLATA, SEP) are required for floral organ determination in all four whorls. ABCE proteins can interact physically with each other to form higher-order protein complexes and bind to the CArG-box motifs of downstream genes to regulate their transcription.

Almost all ABCE genes encode MIKC-type MADS-box transcription factors, with the only exception being AP2. These proteins consist of a highly conserved MADS domain for DNA binding, a K domain for protein interaction, and a less conserved intervening I region and C-terminal region. Class E genes form a small family in different plant species. For instance, there are four SEP members in Arabidopsis, namely, SEPI–4. Their single mutants exhibited subtle phenotypes, but the floral organs could be converted into sepal or leaves in the triple or quadruple mutants, indicating that they play largely redundant roles in flower development.

After pollination, fruits usually develop from ovarian tissues, such as tomato (Solanum lycopersicum), or occasionally from nonovary floral tissues, such as the receptacle in strawberry or the hypanthium in apple (Malus domestica). Parthenocarpy is defined as fruit development without fertilization, which is a desired trait to ensure high yields under unfavorable conditions for pollination. In strawberry, the hormones auxin and gibberellic acid (GA) play important roles in parthenocarpy. In addition, homeotic genes in flower development also take part in controlling fruit development. For example, reduced expression of either TM29 (SEP1/2-like) or TMS (SEP3-like) caused parthenocarpy in tomato. The suppression of MdMADS8/9 (SEP1/2-like) in apple led to a loss of flesh tissue developed from hypanthium and a delay in ripening. Some apple varieties develop parthenocarpic fruit owing to transposon insertion in the introns of MdPI. These results indicate that the class B and E genes act as repressors of fruit development when fertilization does not occur.

In F. vesca, the class ABCE genes have been identified according to sequence homology, and their expression patterns exhibit tissue specificity in floral organs, consistent with the supposed regulatory functions during flower development. However, whether they truly play ABCE functions is still unknown. The knockout of FaTM6 in cultivated strawberry, the ortholog of FveAP3, caused the defective development of petals and stamens. The suppression of FvMADS9 (SEP1/2-like) resulted in the green coloration of petals and delayed ripening. Other ABCE gene homologs have not been functionally studied in strawberry.

In this work, we identified an EMS mutant in F. vesca called R27, which developed sepaloid floral organs in the whorls of petals, stamens, and carpels. Additionally, R27 developed fruit without fertilization. Gene isolation revealed a point mutation in FveSEP3 causing a single amino acid G to E conversion in the MADS domain. The identification of the R27 mutant allowed us to unveil the functions of FveSEP3 in flower and fruit development in woodland strawberry.

**Results**

EMS mutant R27 in F. vesca developed sepaloid floral organs in the inner three whorls. The flowers of woodland strawberry F. vesca contain four typical whorls of organs, including 5 sepals, 5 white petals, 20 stamens, and numerous unfused carpels attached to the domed receptacle. To discover essential genes regulating flower morphogenesis, we screened the EMS mutagenized M2 population of the F. vesca variety Ruegen generated as described previously and identified a mutant numbered R27 with defective floral organs (Fig. 1a). Compared to the wild type (WT), the heterozygous R27 developed basically normal flowers, except that the petals had serrated margins. The homozygous R27 mutant fell into two types based on the severity of floral defects. A majority of R27 had type I flowers, and only a small fraction developed type II flowers. Both types of flowers had green leaf-like organs instead of white petals (Fig. 1a). However, the third and fourth whorls exhibited more diverse phenotypes. In type I flowers, the outer stamens became green and flat tissues with serrated margins; the inner stamens still developed into filaments and anthers, but the anthers had deeper dents between the yellow locules and contained green mosaic tissues (Fig. 1b). The carpels of type I flowers had a similar shape to the WT carpels, but the carpel walls became green instead of pale green, and the styles were more pointy (Fig. 1b). In type II flowers, a higher percentage of stamens became green leaf-like tissues with more epidermal hairs, and the carpels completely lost the regular structure and became leaf-like tissues with dense epidermal hairs (Fig. 1b).

To determine the identity of the sepaloid tissues, scanning electron microscopy (SEM) was used to examine the epidermal cells. In the WT, the adaxial and abaxial sides of sepals had epidermal cells with irregular size and shape; additionally, the abaxial side bore stomata and epidermal hairs (Fig. 1c). The adaxial side of petals in the WT had conical cells with straight epicuticular ridges running from the apex to the base, while the abaxial epidermal cells had irregular shapes with wavy epicuticular ridges (Fig. 1c). In contrast, the green tissues converted from petals in type I and II flowers completely lost the features of petal epidermal cells and were similar to sepals on both adaxial and abaxial sides (Fig. 1d). Moreover, type II flowers exhibited internode elongation between the stamen whorl and carpel whorl (Supplementary Fig. S1), suggesting that the entire flower was partially converted into a shoot.
Floral organ morphologies at different developmental stages in R27

To examine the development process of floral organs, flowers of WT and R27 were further observed at different stages by SEM. Only type I flowers in R27 were examined, as they were the main type. At stage 5, the five sepals appeared with long epidermal hairs at the adaxial side, and petal primordia were initiated (Fig. 2). There was no obvious morphology difference between the WT and R27 flowers up to this stage. At stage 7, the petals in the WT arose with smooth margins, the stamens exhibited a slightly lobed structure, and the carpel primordia initiated from the receptacle dome acropetally. In R27, the petals exhibited three serrations with epidermal hairs engendering at the abaxial surface, and the stamen exhibited a slightly lobed structure, and the carpel primordia initiated from the receptacle dome acropetally. In R27, the petals exhibited three serrations with epidermal hairs engendering at the abaxial surface, and the stamen exhibited a slightly lobed structure, and the carpel primordia initiated from the receptacle dome acropetally. At stage 8, all the carpels arose without obvious differences in shape between the WT and R27. At stage 9, the WT carpels exhibited a water prop-like shape with round tips, while the R27 carpels had thin and pointy styles with epidermal hairs emerging. At stages 10 and 11, the WT carpels possessed bifurcated style atop without any hairs, while the pointy styles on the top of R27 carpels grew longer and bore more and longer epidermal hairs (Fig. 2).

R27 was caused by a G to E amino acid mutation in FveSEP3

The R27 mutant was found in the M2 EMS population of the F. vesca variety Ruegen. To identify the causative mutation, the heterozygous R27 mutant was backcrossed with WT Ruegen to segregate away unrelated background mutations. The heterozygous R27 F1 from the backcross was selfed to generate an F2 population, in which a segregation ratio of 23:47:22 (WT:heterozygous:homozygous) was observed. This ratio was fitted to 1:2:1 ($\chi^2 = 1.875; \chi^2_{0.05} = 5.99$), suggesting that a single gene accounted for the striking phenotypes. Young leaves of 20 heterozygous mutants and 20 homozygous mutants were pooled for whole-genome sequencing. Totals of 28.7 million and 30.5 million paired-end reads at 150 bp were obtained for the two groups. Bioinformatics analysis revealed 19 high-quality single-nucleotide polymorphisms (SNPs) after filtering based on our in-house pipeline. Of these SNPs, 18 were located in the exonic regions on
chromosome 4 (Table S1). After filtering, the G to A SNP located in the first exon of FvH4.4g23530, causing amino acid conversion from G (Gly) to E (Glu) at residue position 27, was considered the primary candidate (Fig. 3a). This mutation was further confirmed to be homozygous in 44 F2 mutants by polymerase chain reaction (PCR) amplification and Sanger sequencing of the amplified DNA. Sequence analysis revealed that FvH4.4g23530 shared a high similarity to the MADS-box gene SEP3 in Arabidopsis, belonging to the class E floral homeotic genes10,11,22. Therefore, this gene was named FveSEP3 as previously reported18, and R27 is called fvesep3 hereafter.

Characterization of FveSEP3 and its homologs
To better understand the gene functions, a phylogenetic tree was constructed using the homologs of FveSEP3 in strawberry, Arabidopsis, and tomato (S. lycopersicum). These proteins could be divided into three clades: the SEP1/2 clade, including FveSEP1; the SEP3 clade, including FveSEP3; and the SEP4 clade, including FveSEP4 and FveSEP-Like (Fig. 3b). Sequence alignment revealed that all the FveSEPs had conserved MADS domains and K domains in the typical MIKC-type MADS-box protein (Supplementary Fig. S2). Moreover, their homologs in the cultivated strawberry genome were also identified23,24. There were two FveSEP1 homologs...
(FxaC_21g10230, FxaC_22g08610), three FveSEP3 homologs (FxaC_13g22210, FxaC_15g15520, FxaC_14g14650), and three FveSEP4 homologs (FxaC_17g18090, FxaC_20g14200, FxaC_18g32540) (Supplementary Fig. S2a). The mutation in FveSEP3 was located in the highly conserved MADS domain, as indicated by sequence alignment of 18 FveSEP3 homologs in different plant species (Fig. 3c).

To detect the subcellular localization of FveSEP3, the 3S::FveSEP3-GFP construct was transiently transformed into tobacco leaves mediated by agroinfiltration. The results showed that the FveSEP3-GFP signal was detected in the nucleus and colocalized with the nuclear marker VirD2NLS-mCherry, consistent with its roles in the regulation of transcription (Fig. 3d). Moreover, the expression patterns of the four FveSEP genes were obtained from the transcriptome database generated from a range of vegetative and reproductive tissues of F. vesca. The results demonstrated that FveSEP3 was broadly expressed not only in the floral tissues, such as anthers and carpels, but also in the fruit, including the ghosts (endosperms and seed coats), carpel walls, and especially receptacles (cortex and pith) (Fig. 3e). FveSEP4 was expressed at low levels in flowers and seeds but was more highly expressed in receptacles than FveSEP3. In contrast, FveSEP1 and FveSEP-Like were expressed at much lower levels. According to RNA sequencing (RNA-seq) data generated from cultivated strawberry, the three FveSEP3 homologs were also most abundantly expressed in both achenes and fruit receptacles compared to other
genes (Supplementary Fig. S2b). These expression patterns indicated that FveSEP3 and FveSEP4 might play important roles in both flowers and fruit in strawberry.

The fvesep3CR mutants exhibited phenotypes similar to fvesep3

R27/fvesep3 exhibited a semidominant inheritance habit, making it hard to define gene functions. To clarify this question, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9)-mediated genome editing was performed to knock out FveSEP3 in the F. vesca strain Hawaii 4 (H4). Two FveSEP3-specific single-guide RNA (sgRNA) target sites were designed in one construct. A total of ten independent transgenic lines were obtained and confirmed by the amplification of the Cas9 and GFP fragments (Supplementary Fig. S3). All of them developed aberrant flowers in the T0 generation, indicating a high editing efficiency. Then we examined the induced mutations at the two target sites by cloning and Sanger sequencing in the two lines. In Line 1, a proportion of the clones from the sgRNA1 target site harbored a 1-bp deletion, while all the clones from sgRNA2 harbored either 2- or 4-bp deletions (Fig. 4a). In Line 2, the sgRNA1 target site harbored both point mutation (C to T) and WT alleles, but all of the clones from the sgRNA2 target site were mutated with short deletions. These mutations resulted in frameshift and truncated FveSEP3 in both fvesep3CR lines. No editing was found in the other three FveSEP genes by sequencing. Furthermore, the expression level of the FveSEP3CR mutant transcripts was greatly reduced in the fvesep3CR mutant lines (Supplementary Fig. S4), suggesting that FveSEP3 might be null in these lines.

The two fvesep3CR mutant lines exhibited similar alterations in flowers to each other and to type 1 fvesep3. Specifically, the petals and stamens became sepaloid organs (Fig. 4b). The carpels were similar to WT, consisting of two parts, but the styles became shorter and

![Fig. 4 Generation and characterization of fvesep3CR mutants in Fragaria vesca. a The top panel shows the location and sequences of two guide RNAs (sgRNA1 and sgRNA2) used to generate mutations in the FveSEP3 gene. The sgRNA target sites in two T0 mutant lines, fvesep3CR L1 and L2, were analyzed to show sequence variations examined with the primers indicated by magenta arrows. The sgRNA is indicated with red. The mutated nucleotides are indicated with blue, and deletions are indicated with dashes. For m/n, n indicates the number of clones examined, and m indicates the number of bacterial colonies showing the indicated sequence. b Images showing the whole flowers and dissected floral organs of the wild-type (WT) F. vesca variety H4 and its two gene-edited lines fvesep3CR L1 and L2. c SEM images showing the stage 12 flowers of WT and fvesep3CR L1 and L2. Stamens and carpels are false-colored. d Images showing the seeds and embryos of WT and fvesep3CR L1 and L2. Scale bars: b 0.5 cm for flowers and petals, 1 mm for stamens and carpels, c 5 mm, d 1 mm for seeds, 0.5 mm for embryos.
thinner with dried tips. Closer observation revealed a striking similarity between \textit{fvesep3} and type I \textit{fvesep} at stage 12 flowers by SEM, such as the pointy and hairy carpels (Fig. 4c). Dissection of the achenes at 30 days post anthesis (DPA) revealed no seeds inside, suggesting failure in fertilization (Fig. 4d). These results supported that \textit{FveSEP3} plays predominant roles in floral organogenesis among the E genes.

The floral homeotic genes were differentially expressed in \textit{fvesep3}

In Arabidopsis, SEP3 can directly bind to the promoters of thousands of downstream genes to regulate their expression levels, including ABCE floral homeotic genes. To compare the expression levels of these genes, flower buds at stages 9–10 of WT and \textit{fvesep3} were used for quantitative reverse transcriptase (qRT)-PCR. The two class A genes, \textit{FveAP1} and \textit{FveAP2}, were significantly increased in \textit{fvesep3}, while the class B and C genes (\textit{FveAP3}, \textit{FvePla}, and \textit{FveAG}) were downregulated in \textit{fvesep3} (Fig. 5a), which is consistent with the conversion of petals and stamens into sepaloid organs and leaf-like carpels. The \textit{FveSEP3} transcript itself is significantly upregulated in \textit{fvesep3}, perhaps due to feedback regulation at the transcription level. This might result in high levels of the \textit{FveSEP3} mutant protein in \textit{fvesep3}. Interestingly, two class E genes (\textit{FveSEP-Like} and \textit{FveSEP4}) were also dramatically upregulated in \textit{fvesep3} (Fig. 5a), suggesting either direct transcriptional regulation or a transcriptional compensation effect.

\textbf{FveSEP3 physically interacts with the ABCE class of MADS-box proteins}

The class ABCE proteins could physically interact with each other to form higher-order protein complexes. To investigate whether strawberry ABCE class MADS-box proteins interact among themselves, a yeast two-hybrid assay was performed. The results showed that \textit{FveSEP3} strongly interacted with all ABCE class MADS-box proteins except \textit{FveAP3} (Fig. 5b, c and Supplementary Fig. S6a). These interactions were also verified by the split-luciferase assay (Fig. 5d). The fluorescence intensity in the combination of \textit{FveSEP3} and \textit{FveAP3} was much lower than that in the other combinations, indicating that they interacted with each other more weakly. These results suggested that the strawberry SEP3 protein is able to interact with the A, B, C, and E classes of proteins.

\textbf{\textit{FveSEP3}G27E overexpression caused sepaloid floral organs in Arabidopsis}

Genetic studies revealed that the \textit{fvesep3}/R27 mutant exhibited a semidominant inheritance habit. To determine the functions of \textit{FveSEP3}G27E, WT \textit{FveSEP3} and \textit{FveSEP3}G27E were overexpressed, driven by the 35S promoter in WT Arabidopsis. Twenty independent \textit{FveSEP3-ox} transgenic lines were obtained in the T1 generation, all of which looked like the WT. For \textit{FveSEP3}G27E overexpression, 15 out of 44 transgenic lines (34%) exhibited defective floral organs in the T1 generation (Supplementary Fig. S5a). Compared to the two \textit{FveSEP3-ox} lines (L19 and L15), \textit{FveSEP3}G27E was expressed at a similar level in L1 and a much lower expression level in L2, as examined by qRT-PCR (Supplementary Fig. S5b). Close observation revealed that the sepals of \textit{FveSEP3}G27E-ox L1 became leaf-like organs and much larger in size, that the petals and stamens became sepaloid organs, and that the gynoecium was replaced by a stalked carpel (Supplementary Fig. S5c). These results indicated that the flower defects in \textit{FveSEP3}G27E-ox were not caused by higher expression levels compared to \textit{FveSEP3-ox}.

The \textit{FveSEP3}G27E-ox phenotype resembled that of the \textit{sep1/2/3} mutants in Arabidopsis, indicating dysfunction in the Arabidopsis SEPs because of the introduction of \textit{FveSEP3}G27E. To test the underlying mechanism, we examined the interactions between \textit{FveSEP3}G27E and other ABCE class MADS-box proteins by yeast two-hybrid assay. Similar to \textit{FveSEP3}, \textit{FveSEP3}G27E could also interact with strawberry ABCE class MADS-box proteins (Supplementary Fig. S6a, b), which is in line with the intact K domain responsible for interaction. In contrast, \textit{FveSEP3CR} lost its interaction capacity with \textit{FvePlb} and showed reduced interaction with \textit{FveSEP1} and \textit{FveSEP4} (Supplementary Fig. S6c). Therefore, we speculated that \textit{FveSEP3}G27E might be a dominant-negative allele.

\textbf{FveSEP3 mutations caused parthenocarpic fruit and delayed fruit ripening}

We noticed that both \textit{fvesep3} and \textit{fvesep3CR} flowers exhibited receptacle enlargement, even though no fertilization occurred due to defective floral organs. To better characterize the fruit phenotype, fruit morphologies were observed from anthesis to full ripening in WT Ruigen and \textit{fvesep3} (both red-ripened). The WT fruit enlarged gradually after fertilization, reached the turning stage at 25 DPA, and became mature at 30 DPA, as determined by the size and color (Fig. 6a). In contrast, the \textit{fvesep3} fruit enlarged more slowly but finally ripened at 65 DPA, showing red pigments in both achenes and receptacles (Fig. 6a, b). Fruit growth was also observed in the \textit{fvesep3CR} mutants and the WT control YW (both white-fruited), showing similar trends to \textit{fvesep3} (Fig. 6c, d). These results indicated that \textit{FveSEP3} acts as a repressor of receptacle enlargement in the absence of fertilization and a promoter of fruit ripening.

\textbf{Functions of \textit{FveSEP3} in fruit initiation}

To identify the roles of \textit{FveSEP3} in fruit initiation, transcriptome analysis was performed for the entire
young fruit (achenes and receptacle) at 6–7 DPA of fvesep3, the pollinated, and unpollinated WT fruit (Fig. 7a). Three biological replicates were harvested for each sample. Finally, a total of 29.4–43.4 million reads were obtained for each RNA-seq library with a high alignment percentage to the genome (>94%) (Table S2). Principal component analysis (PCA) revealed that the three biological replicates for each sample clustered closely together, while the samples from different materials were separated far away (Supplementary Fig. S7). Pairwise comparisons were carried out to identify differentially expressed genes. When fvesep3 was compared to WT-Unpol, 1,959 genes were significantly upregulated, and 1,922 genes were significantly downregulated in fvesep3.
when WT-Pol was compared to WT-Unpol, 1,474 genes were significantly upregulated, and 869 genes were significantly downregulated (Data S1). Among these genes, there were 332 common upregulated genes and 418 common downregulated genes (Fig. 7b). Among the upregulated genes in fvesep3 compared to WT-Unpol, the first three enriched gene ontology (GO) terms were related to photosynthesis, in line with the dark green color of fvesep3 achenes (Fig. 7c). In both upregulated gene lists, there were also several common enriched GO terms on the cell wall and others, suggesting that similar biological processes were involved during fvesep3 and pollinated WT fruit development.

Auxin and GA are the key hormones promoting fruit initiation in strawberry. A total of 19 genes in the auxin pathway, including biosynthesis, transport, metabolism, and signaling, were differentially expressed in the pairwise comparisons (Table S3). Some of these genes were upregulated in both fvesep3 and WT-Pol compared to WT-Unpol, such as FveYUC10, FveLAX1, FveGH3.17, and FveARF7 (Fig. 7d). Some genes were upregulated in only one comparison; for example, FveARF13, FveIAA4, FveIAA7, FveIAA21, FveIAA24, and FveIAA26 were specifically induced in WT-Pol, while FvePIN5, FveGH3.18, and FveIAA20 were specifically induced in fvesep3. A small number of auxin genes were downregulated in both fvesep3 and WT-Pol, such as FveLAX2 and FveARF2. In addition, eight genes in the GA pathway were identified as differentially expressed genes, including the FveGA3ox and FveGA2ox genes responsible for GA biosynthesis and homeostasis (Fig. 7d). In the signaling pathway, the GA receptor gene FveGID1b was downregulated in both fvesep3 and WT-Pol. To confirm the results, the expression levels of 13 auxin genes were examined by qRT-PCR in the same tissues used for RNA-seq analysis. A majority of the genes exhibited the same expression patterns as the RNA-seq data (Fig. 7e). Together, the fvesep3 fruit employed similar pathways but sometimes different genes for growth in comparison with the pollinated WT fruit.

**Discussion**

Flower is an important organ for fruit production. The Rosaceae family contains several fruit crops of high economic value worldwide, such as apple, peach, pear, cherry, and strawberry. As most of them are woody trees, the genetic control of flower development is less studied in these species. ABCE homeotic genes play important roles in floral organ specification. In this study, different alleles of the E class gene FveSEP3 were generated in F. vesca by EMS mutagenesis and CRISPR/Cas9. Taking advantage of
these valuable mutants, we revealed that *FveSEP3* plays critical roles in both flower and fruit development.

**FveSEP3 plays predominant E functions in flower development among FveSEPs**

The *F. vesca* genome contains four *SEP* genes based on sequence similarities. According to the transcriptome data, *FveSEP3* and *FveSEP4* were more abundantly expressed than the other two genes (Fig. 3e).

The *fvesep3<sup>CR</sup>* mutants, in which the function of *FveSEP3* should have been completely abolished due to frameshift and greatly reduced expression, exhibited severe defects in floral organ specialization, suggesting a predominant role of *FveSEP3* in flower development compared with other *FveSEPs*. This differs from the functional redundancy of the *SEP*s in Arabidopsis. The point mutation in the EMS mutant R27/fvesep3 was able to convert all floral organs to leaf-like structures in the type II flowers (Fig. 1).
and Supplementary Fig. S1), a stronger phenotype than the type I flowers and the fvesep3CR mutants. This may imply that strawberry SEPs also possess redundant roles but to a lesser extent than Arabidopsis homologs.

The heterozygous R27/fvesep3 flowers developed petals with serrated margins (Fig. 1), and the homozygous flowers were separated into two types, suggesting that the R27 allele of FveSEP3 is semidominant or dosage-dependent. When FveSEP3 was overexpressed in Arabidopsis, the WT version did not cause an obvious phenotype (Supplementary Fig. S4), which was different from the AtSEP3-ox plants showing early flowering with solitary flowers and the early termination of inflorescences. However, the overexpression of the mutant version FveSEP3G27E led to the conversion of petals and stamens to sepaloid organs, resembling the sep1/2/3 triple or sep1/2/3/4 quadruple mutants in Arabidopsis. Based on these observations, we hypothesize that fvesep3G27E might be a dominant-negative allele. The FveSEP3G27E mutation is located in the conserved MADS domain, which is responsible for DNA binding. Consistently, FveSEP3G27E preserved the interaction capacity with other ABCE class MADS-box proteins (Supplementary Fig. S6). A previous study found that the highly conserved arginine residue at position 3 contributes significantly to the binding specificity. Our mutation occurred at another highly conserved amino acid G at position 27 (Fig. 3a). Taken together, FveSEP3G27E is able to form a complex with other proteins, but the complex might have lost or reduced the DNA-binding capacity to downstream genes, thereby causing the dominant-negative effect.

In addition to regulating floral organ specialization, SEP genes also take part in the maintenance of floral meristem identity in other plant species. The cosuppression of the SEP3 homolog FBP2 (Floral Binding Protein 2) in petunia resulted in the development of ectopic inflorescences. A lack of SEPs could induce the formation of secondary flowers in Arabidopsis. The reduced expression of the SEP1-like gene STM29 caused the growth of ectopic shoots. In contrast, no change in floral meristem determinacy was observed in either R27 or fvesep3GR, indicating that strawberry SEPs may have lost this function.

The class B and C genes were significantly downregulated in the flower buds of fvesep3 (Fig. 5a), consistent with the loss of petal and stamen identity. A previous study revealed that AtSEP3 could directly bind to the promoter regions of the B and C genes. As functional evidence, the severe AtSEP3-ox lines elicited the homeotic transformation of sepal and inflorescence meristems into carpeloid structures, indicating the activation of class B and C functions. It is possible that the class B and C genes in strawberry might also be directly regulated by FveSEP3. Alternatively, expression level changes of the A, B, and C genes in fvesep3 might also be an indirect effect of the conversion of stamens and carpels to sepalids. The ABCE class of MADS-box proteins directly interacts with each other to form tetrameric complexes and then binds to two neighboring CArG-box motifs of downstream genes. The strong interactions between FveSEP3 and all ABCE class MADS-box proteins (except for the weak interaction with FveAP3) examined by both yeast two-hybrid and split luciferase assays indicate that this mechanism is also conserved in strawberry (Fig. 5b–d).

FveSEP3 is essential for fruit growth and ripening

The fvesep3 mutants obtained by either EMS mutagenesis or genome editing in F. vesca exhibited the development of parthenocarpic fruit, which provides convincing evidence on the SEP genes repressing fruit growth in strawberry. The three FaSEP3 genes in cultivated strawberry were also highly expressed in the fruit receptacle (Supplementary Fig. 52b), a similar expression pattern to FveSEP3, suggesting their conserved functions in different strawberry species. Several lines of evidence highlight the class B and E genes as key repressors of fruit growth. For instance, the insertion of transposons in the MdPI intron resulted in the development of parthenocarpic fruit in apple germplasms. In contrast, ectopic expression of PI inhibited fruit flesh tissue growth, resulting in fleshless, small berries in both apple and grape (Vitis vinifera). The downregulation of the SEP1-like gene TM29 caused parthenocarpic fruit in tomato. In apple, suppression of the SEP1/2-like MAD8/9 resulted in varying degrees of reduction of cortex tissue, the flesh from hypanthium, indicating a positive role of SEP1/2 in fruit growth. In our results, the receptacles were not enlarged in the type II flowers of R27 (Supplementary Fig. S1), implying the presence of FveSEPs playing opposing roles to FveSEP3 in fruit growth.

Transcriptome analysis revealed that a small fraction of upregulated genes were shared in the young fruit of fvesep3 (16.9%) and pollinated WT (22.5%) compared with the unpollinated WT flower (Fig. 7b), indicating quite different transcriptome landscapes. However, several similar GO terms were enriched among these two gene lists, suggesting that similar biological processes were employed for fruit growth. Auxin is the key hormone regulating fruit initiation in strawberry. We found that a number of genes involved in auxin biosynthesis, transport, and signaling were differentially expressed, such as the ARF and IAA genes (Fig. 7d, e). Expansin (EXP) and small auxin upregulated RNA (SAUR) genes act downstream of auxin signaling to induce cell expansion. We found several differentially expressed EXP and SAUR genes in the comparisons, and a majority of them were specific to only one sample (Data S1). Additionally, several GA pathway genes were also differentially expressed (Fig. 7d), consistent with the important roles of GA in early fruit development in strawberry. A feature
here is that different members in the same gene family were adopted by either fvesep3 or pollinated WT fruit, which may explain the lower percentage of shared genes.

The expression level of FveSEP3 was increased in fruit from the green to the turning stage (15D vs. 22D) according to the transcriptome data, suggesting a possible role of FveSEP3 in fruit ripening (Fig. 3e). Indeed, fruit ripening in fvesep3 was significantly delayed. The SEP genes in other clades are also responsible for fruit ripening in different species. For instance, silencing the SEP1/2-like gene FaMADS9 in cultivated strawberry resulted in retarded fruit maturation. Suppressed expression of the SEP1/2-like genes PrupeSEP1 in peach and PpMADS7 in sweet cherry by virus-induced gene silencing delayed fruit ripening and softening. Similarly, reduced expression of the SEP1/2-like genes MaMADS1 or MaMADS2 showed delayed ripening and longer shelf life in banana. The well-known SEP1-like gene SIMADS-RIN (RIN) in tomato has been identified as a master regulator of fruit ripening. In addition, the class C homeotic gene PrpPLENA is upregulated during ripening in peach, and the overexpression of this gene in tomato accelerates fruit ripening. These results indicate that floral homeotic genes are broadly involved in the regulation of fruit ripening. In the climacteric fruit tomato, RIN directly regulated the expression of key enzymes of ethylene biosynthesis during fruit ripening control. PrpSEP1 can bind to the promoter of the pollen wall softening enzyme polygalacturonase. Strawberry fruit belongs to the no climacteric type of ripening that is primarily regulated by the ABA pathway. How FveSEP3 interacts with the ABA pathway or other associated genes during fruit ripening awaits further investigation.

**Materials and Methods**

**Plant materials and growth conditions**

Three F. vesca accessions, Ruegen (red fruited), Yellow Wonder, and Hawaii 4 (white fruited), were used as the WT in this study. Both woodland strawberry and Arabidopsis plants were grown in a growth room under a light and 8 h dark at 22 °C.

**Gene isolation of the EMS mutant R27**

Heterozygous R27 was crossed with WT Ruegen; F1 heterozygous R27 plants were selected and allowed to self-cross to generate an F2 population. Equal amounts of young leaves were pooled from 20 F2 homozygous and 20 F2 heterozygous mutants. Genomic DNA was extracted using a CTAB method. Genome sequencing was performed using the Illumina HiSeq X Ten platform (Novogene, Beijing) and analyzed as described previously. The candidate mutation was examined among individual F2 mutants by cloning and Sanger sequencing.

**Scanning electron microscopy**

Samples were fixed in 2.5% glutaraldehyde at 4 °C overnight; washed with phosphate-buffered saline (PBS; 0.1 M, without NaCl) 3–5 times (15 min each time); fixed for 2–3 h in osmium acid; washed with PBS (0.1 M, without NaCl) 3–5 times (15 min each time); treated sequentially with 30, 50, 70, 80, 90, 95, and 100% ethanol for 15 min each; transferred to isoamyl acetate 3 times (20 min each time); critical point dried; coated with gold for 45 s; and photographed under a scanning electron microscope (JSM-6390LV).

**Phylogenetic analysis**

The protein sequences were obtained from GDR for F. vesca (rosaceae.org), TAIR for Arabidopsis (Arabidopsis.org), and Sol Genomics Network for tomato (solgenomics.net). An unrooted phylogenetic tree was constructed using MEGA7 with the neighbor-joining statistical method and bootstrap analysis (1000 replicates).

**Plasmid construction**

Genomic DNA or cDNA derived from RNA extracted from flowers or young leaves of Ruegen or R27 was used for sequence amplification. For overexpression, the full-length coding sequences of FveSEP3 and FveSEP3G27E were cloned into pENTR1A and inserted into the binary vector pK7WG2D. For genome editing, two FveSEP3-specific sgRNAs were designed using the web server CRISPR-P2.0 (http://crispr.hzau.edu.cn/CRISPR2/). Two AtU6 promoter-sgRNA-AtU6 terminator cassettes were amplified by PCR using pCBC-DT1T2 as the template and then inserted into pHSE401G by Golden Gate Assembly and confirmed by Sanger sequencing. For subcellular localization, the coding sequence of FveSEP3 was cloned into the binary vector pRI101 at the EcoRI and NdeI sites and fused with GFP by the Gibson cloning method. The primers are shown in Table S4.

**Stable transformation in woodland strawberry**

Woodland strawberry transformation was carried out as described previously. The CRISPR/Cas9 construct was transformed into the F. vesca variety H4. During transformation, positive transgenic calli and regenerated plants were selected using both hygromycin (4 mg L⁻¹) and GFP fluorescence, which was examined under a fluorescence dissecting microscope (Microshot Technology Ltd, Guangzhou, China, MZX81).

**Stable transformation in Arabidopsis**

Arabidopsis Col-0 was transformed with FveSEP3-ox and FveSEP3G27E-ox in Agrobacterium tumefaciens GV3101 using the floral-dip method. The T1 transgenic lines were selected on half-strength MS (M5524, Sigma-Aldrich) with 100 mg L⁻¹ kanamycin.
**Subcellular localization analysis**
Agrobacterium colonies of *FveSEP3-GFP* and the nuclear marker *VirD2-NLS-mCherry* were grown in 2 ml of liquid LB medium at 28 °C overnight. The culture was then spun down and resuspended in buffer (5 g l⁻¹ D-glucose, 50 mM MES, 2 mM Na₃PO₄, 100 μM acacetosyringone, pH 5.6) to reach an OD₆₀₀ of 0.3. Equal volumes of the two suspensions were mixed together and infiltrated into the leaves of 3-week-old tobacco (*Nicotiana benthamiana*) using syringes. After 2 days of infiltration, images of GFP fluorescence were taken using a confocal laser scanning microscope (TCS SP8; Leica Wetzlar, Germany).

**Transcriptome sequencing and data analysis**
Total RNA was isolated from the entire fruit (achenes and receptacle) at 6–7 DPA. Each sample had three biological replicates. Sequencing was performed on an Illumina NovaSeq platform (Shanghai Personal Biotechnology Co., Ltd, China). The *F. vesca* reference genome with the ver4.0.a2 annotation was downloaded from the GDR website (www.rosaceae.org). Raw reads were first trimmed 9 bp at the 5’ end using Trimmomatic and then mapped to the reference genome using the program hisat2. FeatureCounts was used to count the reads mapped to the gene models. Gene expression levels were calculated as the TPM (transcript per million reads) with Tbtools. Differential gene expression analysis was implemented by the R package DESeq2 and those with an adjusted P value of <0.05 and fold change >2 were considered differentially expressed. PCA was performed using the R package factoextra (ver1.0.7) based on log₂-transformed TPM values. Venn maps were drawn using MeV 4.9.0.

**Quantitative RT-PCR**
Total RNA was isolated from different tissues using a HiPure Plant RNA Mini Kit (Magen, Guangzhou, China; cat no. R4151) and treated with DNase I (Promega) according to the manufacturer’s instructions. Approximately 1 μg of total RNA was used for cDNA synthesis using a PrimeScript™ RT Reagent Kit (TaKaRa, Shiga, Japan; cat no. RR047A). For qPCR, a total volume of 10 μl reaction mixture was set up containing 5 μl of 2× SYBR Green PCR master mix (Catalog # 172–5124, BioRad), 1 μl of 5× diluted cDNA, 0.25 μl of each primer, and 3.5 μl ddH₂O. Amplification was performed using a Quant Studio 7 Flex system (Applied Biosystems, Waltham, MA, USA). The expression level of each gene was calculated using the 2^−ΔΔCT method. FvH4_1g05910, the homolog of CDC27 in the anaphase-promoting complex, and the actin gene At3G18780 were used as the internal controls. All analyses were repeated, and each sample included three biological replicates.

**Yeast two-hybrid assays**
Yeast two-hybrid assays were performed using the Matchmaker™ Gold Two-Hybrid System (Clontech) following the manufacturer’s instructions. The coding sequences of the woodland strawberry ABCE genes were amplified from the cDNA of young WT flowers and inserted into the BD vector pGBKTK7 or the AD vector pGADT7. Combinations of BD and AD vectors were cotransformed into the yeast (*Saccharomyces cerevisiae*) strain AH109. The transformants were selected on SD-Leu-Trp plates (DDO). The interactions were tested on SD-Leu-Trp-His-Ade plates (QDO). At least six individual clones were analyzed.

**Split-luciferase assays**
Full-length *FveSEP3* was cloned into the JW771-nLUC vector. The full-length ABCE genes were cloned into the JW772-cLUC vector. The constructs were transfected into *A. tumefaciens* strain GV3101, and different combinations at a ratio of 1:1 at an OD₆₀₀ of 0.5 were coinfiltrated into tobacco (*N. benthamiana*) leaves. After 2 days, the tobacco leaves were sprayed with 100 mM luciferin, kept in the dark for 10 min, and observed under a low-light cooled charge-coupled device imaging apparatus (LB985 NightSHADE).

**Statistical analyses**
Statistical analyses were performed using SPSS v22.0 (IBM Corp., Armonk, NY, USA). Pairwise comparisons were determined using Student’s *t* test (***, *P* < 0.01).

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**Author contributions**
C.K. and M.P. conceived and designed the experiments; M.P. performed most of the experiments; L.C., R.Z., and Z.C. helped with the experiments; S.H. analyzed the RNA-seq data; M.P. and C.K. wrote the manuscript; Z.L. and J.-L.Y. revised the manuscript. All the authors have read and approved the paper.

**Data availability**
Raw reads of the transcriptome data have been submitted to the Sequence Read Archive at NCBI under the accession number PRJNA718919. The loci of strawberry and Arabidopsis genes or accession numbers of the genes in other species in NCBI are as follows: gene04229/FvH4_4g46420 (FveSEP1), gene04563/FvH4_4g29610 (FvSEP2), gene07201/FvH4_4g23530 (FvSEP3), gene26118/FvH4_5g13510 (FveSEP4), Arabidopsis: AT3G15800 (SEP1), AT1G24260 (SEP2), AT1G24260 (SEP3), AT2G0371 (SEP4); *Solanium lycopersicum*.
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