RESEARCH PAPER

Identification and isolation of a homolog of *AtSEF* gene from apple (*Malus domestica*)

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Abstract

Flowering in fruit trees like apple with a long juvenility is hindered and delayed by some inhibitor genes until their down-regulation by their controlling pathways. Shortening juvenility period could be achieved through identifying and silencing these flowering inhibitors via biotechnological methods. *SEF* is an Arabidopsis homolog of the yeast SWC6 protein, which is a conserved subunit of the SWR1/SRCAP chromatin-remodeling complex probably involved in the substitution of histone H2A and hence down-regulation of *FLOWERING LOCUS C* and *MADS-AFFECTING FLOWERING 4* and increasing *FLOWERING LOCUS T* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* transcript levels. Here, we report the identification of three paralogs of *AtSEF* gene in apple (*Malus domestica*) which may have the same regulatory function in the genetic network of flowering in apple. One of these paralogs, *MdSEF1*, was isolated, cloned and further analyzed through bioinformatic approaches. Amino acid sequences similarity of thisgene to its counterparts from other species showed that it is most similar to a protein from diploid strawberry. In silico analysis of the expression pattern of the isolated gene revealed that it is expressed in young shoot, fruit and root.

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Introduction

Flowering in fruit trees such as apple is of great economic importance. Yield depends on the number and quality of flower buds formed. Flowering is a complicated developmental process of physiological and morphological stages under control of a number of external signals and internal factors. All of these factors are controlled by a complex network of genes. Understanding the processes regulating the phase transition from vegetative to reproductive growth is important in fruit trees because they have a long juvenile period without reproductive development(Thomas and Vince-Prue, 1997). Shortening the juvenile phase is an important goal for fruit tree breeders. About 80 genes involved in the flowering process. *LFY* and *FT* are important genes that stimulate flowering. In contrast to these genes, others genes have inhibitory effects such as *FLC,SE, SEF*. *SEF* promotes *FLC* expression and hence represses Arabidopsis flowering. The purpose of this research is to identify and isolate *SEF* in apple as one of putative flowering repressors.

Plants go through a series of phase transitions throughout their life cycle in response to endogenous and environmental signals. One of them is the transition from vegetative to reproductive phase. Beforehand, the transition fromthe juvenile to adult phase takes placeduring the vegetative phase. In the juvenile phase, plants are not yet competent to undergo reproductive development and do not respond to photoperiod or vernalization. The duration of juvenile phase is different from species to species, with a range of few days, for herbaceous and annual plants, to more than decades (Thomas and Vince-Prue, 1997). After reaching to the adult phase, plants getcompetency to react to floral inducers, which is essential for the transition from vegetative to reproductive phase.This transition to reproductive phase is a key factorin plants reproductive success as well as in their seasonal and geographical adaptation (Matsoukas *et al.*, 2012). In Arabidopsis, there are four independent pathways of signal transduction, i.e. the vernalization, autonomous, and gibberellin and light-dependent pathways(for reviews, see Koornneef,

1998). The integrator genes, such as *FT,SOC1* and *FLC* transmit these signals to the floral meristem identity genes *AP1* and *LFY* at the apical meristems (for reviews, see Michaels, 2009).

FLC encodes a MADS-box transcription factor and is widely expressed in the meristem and leaves (Noh and Amasino, 2003; Sheldon *et al.*, 2002). Regulation of *FLC* expression involves epigenetic control of the functional states of its chromatin by multiple factors (Amasino, 2004; Baurle and Dean, 2006). Expression pattern of*FLC* has revealed that *FLC* expression is needed for the full repression of flowering (Searle *et al.*, 2006). High expression of *FLC*represses at least two floral pathway integrators,*FT* and *SOC1,*and prevents the meristem from being competent to respond to inductive floral signals;while, the vernalization and autonomous pathways promote flowering by repressing *FLC* expression (Hepworth *et al.*, 2002; Lee *et al.*, 2000; Michaels and Amasino, 1999; Michaels *et al.*, 2005; Sheldon *et al.*, 2000). Spatial and temporal expression analysis of *FLC* has shown itsdouble roles in repressing flowering. *FLC* down-regulates *FT* in the leaves and prevents the transport of FT protein from the leaves to the meristem, and *FLC* also inhibits the expression of *SOC1* and the *FT* cofactor *FD* and hence impairs the response of the meristem to the flowering signals (Abe *et al.*, 2005; Corbesier *et al.*, 2007; Searle *et al.*, 2006; Wigge *et al.*, 2005). *FLC* probably playssome other roles in the floral transition, such as lengthening the temperature compensation period specifically at 27°C, possibly through the transcription factor*LUX ARRYTHMO* (*LUX*) (Edwards *et al.*, 2006; Hazen *et al.*, 2005; Swarup *et al.*, 1999).

Serrated Leaves and Early Flowering (SEF) is an Arabidopsis (*Arabidopsis thaliana*) homolog of the yeast SWC6 protein, of which loss-of-function mutants present a pleiotropic phenotype featured by serrated leaves, flowers with altered number and size of organs, frequent lack of inflorescence internodes, and bushy feature.*sef* plants flower earlier than wild-type plants both under inductive and non-inductive

photoperiods. This correlates with strong decrease of *FLC* and *MAF4* transcript levels and increase of *FT* and *SOC1* gene expression.Based on these results, it is postulated that SEF gene, a conserved subunit of the SWR1/SRCAP complex, is involved in up-regulating the FLC gene expression (Rosana *et al.*, 2007) (Fig 1).

Flowering in fruit trees, like apple, has a great economic importance. Yield depends on the number and quality of flower buds formed (Hanke *et al.*, 2007). Apple (*Malus* spp.), belonging to the family Rosaceaeand subfamily Maloideae, is one of the most important fruit tree crops in the world. Nevertheless, the fairly long juvenile phase of apple, which normally lasts from 4 to 8 years or more, makes its breeding process slower (Hackett, 1985). For example, the 'Fuji' apple (*Malus domestica*) from 'Ralls Janet' × 'Delicious', one of the most widespread apple cultivars (O'Rourke *et al.*,2003) has had its first fruit set 12 years after planting (Sadamori *et al.,*1963). Consequently, various practical techniques to accelerate flowering and fruiting of seedlings in the juvenile phase have been considered for years, but not yet completely satisfying. Exploitation of genetic engineering for shortening juvenile period can be carried out through two different approach: overexpression of flower promoting transcription factors and silencing of flower inhibitors. Among these theflowering inhibitor genes,*FLC* and its promoters such as *SEF* can be of great importance, for this purpose. The aim of this research is to identify, isolate and characterize a homolog of*AtSEF* gene from apple as one of the putative flowering repressors.

Materials and methods

In silico analysis

The amino acid sequence of Arabidopsis *AtSEF* gene was used as a query and blasted using BLASTP tool in apple amino acid gene set database of GDR website (http://www.rosaceae.org/). Three similar sequences were identified and designated as *MdSEF1*, *MdSEF2*, and *MdSEF3*. *MdSEF1* was isolated through RT-PCR method as mentioned below. To do *in silico* expression analysis, nucleotide sequence was used as a query to be blasted using BLASTN tool of NCBI website in EST database putting *Malussp.* as the favored organism.

Plant material and RNA extraction

Leaves, stems, flowers and fruits were collected from apple (*Malus domestica* cv. Golden Delicious) trees located inKhalat-PoushanResearch Station of theUniversity of Tabriz. Flowers were collected at full bloom stage and fruits were gathered 35 days after full bloom. All of these tissues were frozen in liquid nitrogen immediately and stored at -80 ℃ until use. Total RNA from leaf, stem and flower tissues was isolated by total RNA purification kit (Bioneer, Korea). Pine tree method was used for RNA isolation from fruit tissue (Chang *et al.*, 1993). RNA quantification was performed by a spectrophotometer at wavelengths of 230, 260 and 280 nm. To confirm the RNA quality, the RNA was electrophoresed on a 1% agarose gel(Fig 2).

RT-PCR

One μg of total RNA was used for cDNA synthesis. The cDNA was synthesized from oligodT(18) primer by reverse transcriptase (MMuLV, Fermentas) considering recommendations of the manufacturer.The*SEF* homolog was amplified using forward and reverse primers, 5'-ttgtacatgaggcaaatctcgactcc- 3' and 5' -tgtttcagcatcaagttcactaaagttacg- 3', respectively. PCR reactions were carried out withone μl of cDNA and Taq DNA polymerase from Fermentasusing an ASTEC-818 (Japan) thermal cycler.The thermal program usedincluded one cycle of primary denaturation at 94 ℃ for 2min; 30 cycles of 94 ℃ for 30s, 45.5 ℃ for 30s and 72 ℃ for 1 min and final extension at 72 ℃ for 10 min.

Cloning of MdSEF1

The amplified fragments of Apple *AtSEF* homolog, *MdSEF1,* with the accession number ofMDP0000767102 wascloned into the pGEM T-Easy vector (Promega) and transformed into DH5alpha E. coli competent cell through one-step method (Chung *et al*, 1989). To ensure that the recombinant DNA

contains the right fragment, colony-PCR was performed using the same primer pair and PCR condition used for gene isolation. Plasmid extraction was done via mini-prep alkaline lysis method (Sambrook *et al.*, 2001). The resulted DNAwas detected on 1% agarose gel (Fig 3).

Phylogenetic analysis

The amino acid sequence of Arabidopsis *SEF* gene was used as a query and blasted using BLASTP tool in apple amino acid gene set database (website www.rosaceae.org). Three similar sequences were identified and designated as *MdSEF1*, *MdSEF2*, and *MdSEF3*.The amino acid sequences from other species similar to AtSEF were extracted from NCBI and were aligned and identity scores were calculated using CLUSTALW alignment tool of MEGA6

software.Neighbor joining method was used for the assessment of sequences distances. Phylogenetic tree were constructed using MEGA6 software.

Results and discussions

Complete RNA coding sequence of *MdSEF1* gene is 1194 bp long. Sequence analysis of this gene showed that this gene has four exons and three introns(Fig 2a).The protein coding sequence of this gene is 515 bp and its deduced amino-acid sequence has 171 residues with a HIT zincfinger domain near its C-terminal end (131-158) (Fig 2b).This protein belongs to a family of transcription factors, which has a HIT zinc finger domain to attach DNA to regulate the expression of its downstream gene that here is supposed to be FLC(Rosana *et al.*, 2007).

Table 1. *In silico*analysis and RT-PCR revealed expression pattern of MdSEF1 gene in different organs of apple plant.

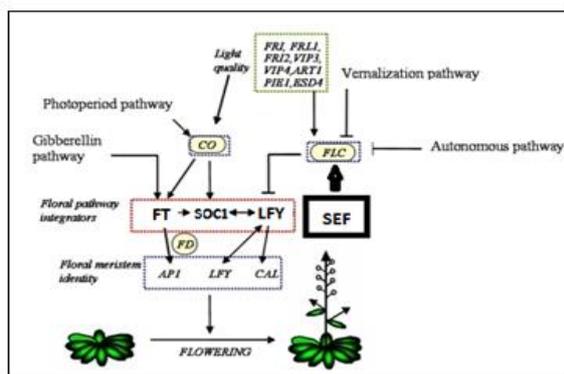| Apple tissue	MdSEF1 Expression	EST GenBank no.	RT-PCR
Young shoot	Yes	CN936515.1	Not certain
Fruit	Yes	CO417698.1	+
Leaf	No	-	-
Root	Yes	GO517882.1	Not evaluated
Flower	Yes	-	+

Fig. 1. Pathways controlling flowering time in *Arabidopsis*. The function of SEF gene has been illustrated. Adapted and Changed from(Corbesier and Coupland,2006).

Zinc finger (Znf) domains are small protein motifs containing multiple finger-like protrusions that make tandem interactions with their target molecule. Some

of these domains bind zinc, but many bind other metals such as iron or no metal at all. For example, some family members form salt bridges to stabilize the finger-like folds. They bind DNA, RNA, protein and/or lipid substrates; nevertheless, they wererecognized first as a DNA-binding motif in TFIIIA, atranscription factor from *Xenopuslaevis* (African clawed frog), however (Klug A, 1999; Hall TM, 2005; Brown RS, 2005;Gamsjaeger *et al.*, 2007; Matthews andSunde, 2002). The amino acid sequence of the finger domains, the linker between fingers, the higher-order structures and the number of fingers determine their binding properties. Most of Znf domains with different binding specificities arefound in clusters.There are many super-families of Znf motifs, whichvary in both sequence and structure. There is a significant diversity in their binding modes, even among members of the same class, illustrating

that stable scaffolds ofZnf motifs have probably evolved for particular functions. Proteins having zinc finger domains play a role in many different processes such as gene expression, trafficking ofmRNA, organization of cytoskeleton, development of epithelial cells, cell adhesion, protein folding, chromatin remodeling and zinc sensing(Laity *et al.*, 2001).The structure zinc-binding motifs are so stable that they don't need any conformational changes when binding their target.
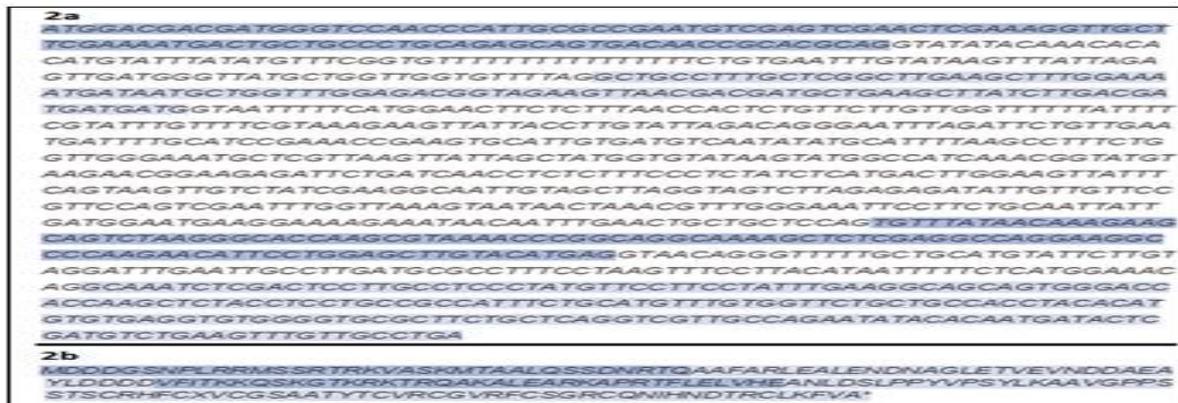

Fig. 2. Nucleotide and deduced amino acid sequence of MdSEF1 gene. In nucleotide sequence (2a), highlighted sequences shows exons and non-highlighted parts illustrates introns. In amino acid sequence (2b), each highlighting color exemplifies exon boundaries.
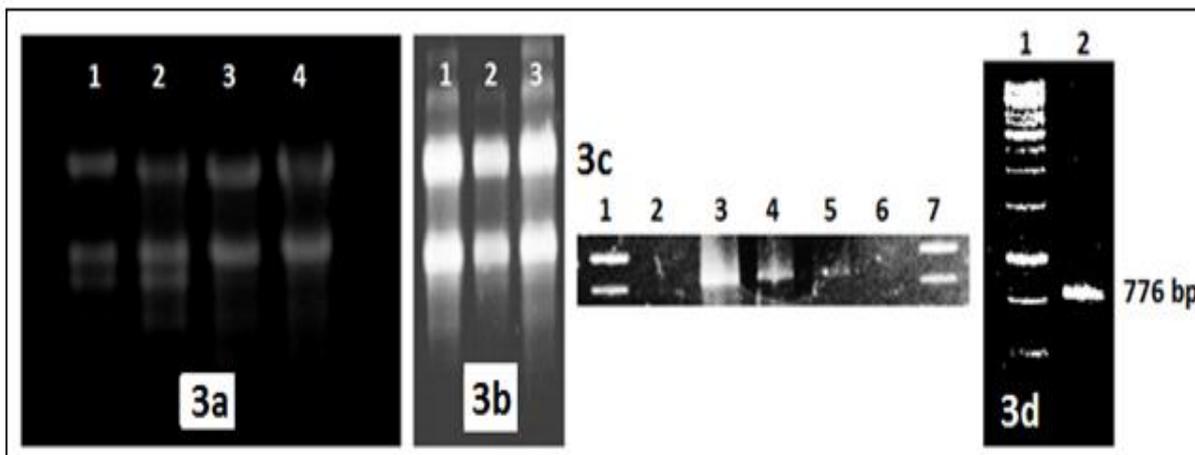

Fig. 3. Electrophoresis on 1% agarose gel: RNA extracted by Pine tree method (3a) for fruit tissue andby RNA Extraction Kit (Bioneer, Korea) (3b) for leaf, stem and flower tissues; 3c) RT-PCR of MdSEF1. Lanes 1 and 7, ladder; 2, empty; 3, 4,fruit; 5, flower and 6, empty.3d) a 776 bp band resulted from RT-PCR of MdSEF1 from flower used for cloning.Lane 1 is ladder and 2 is the PCR product fromflower tissue. O'Gene Ruler 1 kb ladder from Fermentas was used as the ladder.

The HIT-type zinc finger domain, the name of which has been originated from the yeast HIT1protein, can potentially coordinate two zinc atoms with itsseven conserved cysteines and one histidine. (Kawakami *et al.*, 1992).There is some sequence similarities between the HIT-type and MYND-type zinc fingers. The latter is mostly found in nuclear proteins involved in gene regulation and chromatin remodeling, however its function is not yet revealed. This domain is also found in the thyroid receptor interacting protein 3 (TRIP-3) that specifically interacts with the ligand-binding domain of the thyroid receptor. In Arabidopsis, SEF up-regulates FLC, which in turn is a flowering inhibitor integrating vernalization and autonomous pathway signals(*Corbesier and Coupland, 2006*).
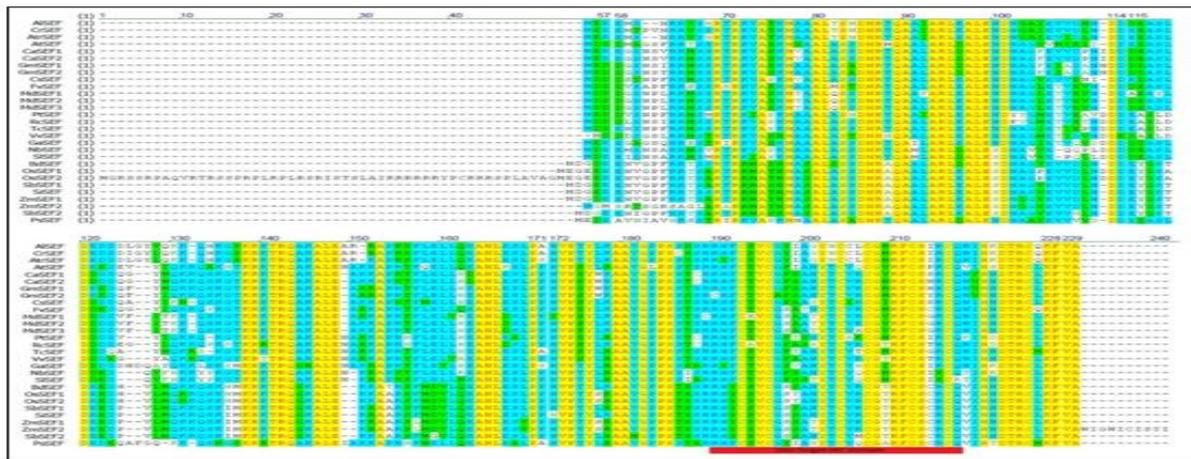
Fig. 4. Alignment of the deduced amino acid sequences of 28 amino acid sequences resulted from MdSEF1 deduced amino acid sequence BLASTp with a hit score more than 200 and a similarity more than 60%. Asterisks indicates amino acid residues of zinc finger HIT domain. Alignment was done using Vector NTI version 9 (Invitrogen). The red bar shows the borders of the identified zinc finger HIT domain found in all of the SEF like amino acid sequences. Abbreviations: Md, Malus domestica; Fv, Fragariavesca; Cs, Cucumissativus; Gm, Glycine max; Tc, Theobroma cacao; Ca, Cicerarietinum; Gm, Glycine max; Ca. Cicerarietinum; Vv, Vitisvinifera; Rc, Ricinuscommunis; Atr, Amborellatrichopoda; Al, Arabidopsis lyrata; At, Arabidopsis thaliana; Pt, Populustrichocarpa; Cr, Capsella rubella; Sb, Sorghum bicolor; Zm, Zea mays; Ga, Genliseaaurea; Os, Oryza sativa; Nt, Nicotianabenthamiana; Sl, Solanumlycopersicum; Bd, Brachypodiumdistachyon; Si, Setaria italic; Ps, Piceasitchensis.
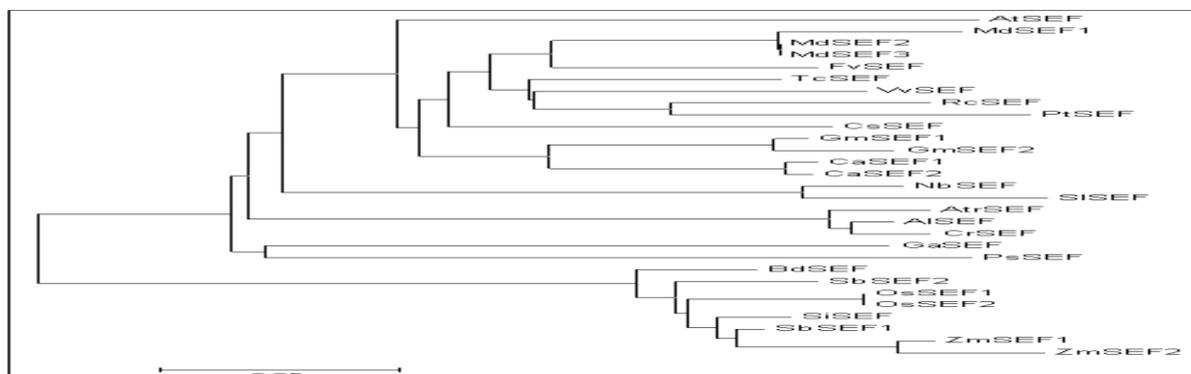

Fig. 5. Evolutionary relationships of taxa: The evolutionary history was inferred using the Neighbor-Joining method(Saitou and Nei,1987). The optimal tree with the sum of branch length = 1.88687468 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 29 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 156 positions in the final dataset. Evolutionary analyses were conducted in MEGA6(Tamura *et al*, 2013). The tree is rootless. Abbreviations: Md, Malus domestica; Fv, Fragariavesca; Cs, Cucumissativus; Gm, Glycine max; Tc, Theobroma cacao; Ca, Cicerarietinum; Gm, Glycine max; Ca. Cicerarietinum; Vv, Vitisvinifera; Rc, Ricinuscommunis; Atr, Amborellatrichopoda; Al, Arabidopsis lyrata; At, Arabidopsis thaliana; Pt, Populustrichocarpa; Cr, Capsella rubella; Sb, Sorghum bicolor; Zm, Zea mays; Ga, Genliseaaurea; Os, Oryza sativa; Nt, Nicotianabenthamiana; Sl, Solanumlycopersicum; Bd, Brachypodiumdistachyon; Si, Setaria italic; Ps, Piceasitchensis.

In silico expression analysis revealed that three tissues of apple including young shoot, root and fruit had an EST in databases (Table1). RT-PCR resulted in a 776 bp band for *MdSEF1*, which were as expected in accordance with designed primers (Fig 3d).Their RNA extraction results on the gel is also observable in Fig 3a and 3d.RT-PCR analysis of apple different tissues demonstrated sharp electrophoretic bandsshowingthe existence of *MdSEF1 mRNA*in fruit and flower, while in other tissues no transcript was detected (Fig 3c). Only the results of RT-PCR and EST analyses for fruit tissue were in agreement with each other. In flower, there was a positive result in RT-PCR, while there was not any reported EST in this tissue. Alignment of 28 amino acid sequences similar to MdSEF1 deduced amino acid sequence has been illustrated in Fig4. In all of the *MdSEF1* like sequences, a HIT zinc finger domain was observed. The phylogenetic analysis of these amino acid sequencesusing MEGA6 illustrated that this protein belongs to the clade two of *SEF* like genes. In this clade, the most similar protein to *MdSEF1*is*FvSEF* from diploid strawberry(Fig 5).Further investigation of the expression pattern of three paralogous *MdSEF* genes is yet to be done.

In Arabidopsis, SEF transcripts was present in all tested tissuesas revealed by semi-quantitativereverse transcription (RT)-PCR experiments.Rosana *et al.* (2007) observed that sef plants flowered in LD with approximately six leaves, whereas wild-type plants flowered with 12 leaves, approximately. Under SD conditions, sef plants flowered with around 21 leaves, while the wild type flowered with about 62 leaves. They showed that SEF probably plays a role in the transition from vegetative to reproductive development.

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