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 this gene 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, 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 from the juvenile to adult phase takes place during 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 get competency to react to floral inducers, which is essential for the transition from vegetative to reproductive phase. This transition to reproductive phase is a key factor in 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 its double 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 plays some other roles in the floral transition, such as lengthening the temperature compensation period specifically at 27°C, possibly through the transcription factor LUX ARRHYTHMO (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 Rosaceae and 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 the flowering 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 to be 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 Malus sp. 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 in Khalat-Poushan Research Station of the University 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 °C 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 oligo(dT)18 primer by reverse transcriptase (MMuLV, Fermentas) considering recommendations of the manufacturer. The SEF homolog was amplified using forward and reverse primers, 5’-ttgtagtcggcaatctgctac-3’ and 5’-tctgatacaagtctcaagtttacg-3’, respectively. PCR reactions were carried out with one µl of cDNA and Taq DNA polymerase from Fermentas using an ASTEC-818 (Japan) thermal cycler. The thermal program used included one cycle of primary denaturation at 94 °C for 2 min; 30 cycles of 94 °C for 30s, 45.5 °C for 30s and 72 °C for 1 min and final extension at 72 °C for 10 min.

Cloning of MdSEF1

The amplified fragments of Apple AtSEF homolog, MdSEF1, with the accession number of MDP0000767102 was cloned 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 DNA was 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.**

<table>
<thead>
<tr>
<th>Apple tissue</th>
<th>MdSEF1 Expression</th>
<th>EST GenBank no.</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young shoot</td>
<td>Yes</td>
<td>CN936515.1</td>
<td>Not certain</td>
</tr>
<tr>
<td>Fruit</td>
<td>Yes</td>
<td>CO417698.1</td>
<td>+</td>
</tr>
<tr>
<td>Leaf</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Root</td>
<td>Yes</td>
<td>GO517882.1</td>
<td>Not evaluated</td>
</tr>
<tr>
<td>Flower</td>
<td>Yes</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**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 were recognized first as a DNA-binding motif in TFIIIA, a transcription factor from *Xenopus laevis* (African clawed frog), however (Klug A., 1999; Hall TM, 2005; Brown RS, 2005; Gamsjaeger et al., 2007; Matthews and Sunde, 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 are found in clusters. There are many super-families of Znf motifs, which vary 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 of Znf motifs have probably evolved for particular functions. Proteins having zinc finger domains play a role in many different processes such as gene expression, trafficking of mRNA, 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 and by 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 from flower 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 HIT protein, can potentially coordinate two zinc atoms with its seven 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, Fragaria vesca; Cs, Cucumis sativus; Gm, Glycine max; Tc, Theobroma cacao; Ca, Cicer arietinum; Gm, Glycine max; Ca. Cicer arietinum; Vv, Vitis vinifera; Rc, Ricinus communis; Atr, Amborella trichopoda; Al, Arabidopsis lyrata; At, Arabidopsis thaliana; Pt, Populus trichocarpa; Cr, Capsella rubella; Sb, Sorghum bicolor; Zm, Zea mays; Ga, Genlisea aurea; Os, Oryza sativa; Nt, Nicotiana benthamiana; Sl, Solanum lycopersicum; Bd, Brachypodium distachyon; Si, Setaria italic; Ps, Picea sitchensis.

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, Fragaria vesca; Cs, Cucumis sativus; Gm, Glycine max; Tc, Theobroma cacao; Ca, Cicer arietinum; Gm, Glycine max; Ca. Cicer arietinum; Vv, Vitis vinifera; Rc, Ricinus communis; Atr, Amborella trichopoda; Al, Arabidopsis lyrata; At, Arabidopsis thaliana; Pt, Populus trichocarpa; Cr, Capsella rubella; Sb, Sorghum bicolor; Zm, Zea mays; Ga, Genlisea aurea; Os, Oryza sativa; Nt, Nicotiana benthamiana; Sl, Solanum lycopersicum; Bd, Brachypodium distachyon; Si, Setaria italic; Ps, Picea sitchensis.
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 bands showing the existence of MdSEF1 mRNA in fruit and flower, while in other tissues no transcript was detected (Fig 3e). 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 Fig 4. In all of the MdSEF1 like sequences, a HIT zinc finger domain was observed. The phylogenetic analysis of these amino acid sequences using 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 MdSEF1 genes is yet to be done.

In Arabidopsis, SEF transcripts were present in all tested tissues as revealed by semi-quantitative reverse 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.

References


http://dx.doi.org/10.1101/gad.373506

http://dx.doi.org/10.1105/tpc.14.19.2527

http://dx.doi.org/10.1046/j.1365-313x.1999.00577.x

http://dx.doi.org/10.1093/molbev/mst197


http://dx.doi.org/10.1126/science.1114358

http://dx.doi.org/10.1016/b978-1-4832-2734-4.50017-6