

Analysis of SFL1 and SFL2 Promoter Region in *Arabidopsis thaliana* using Gateway Cloning System

A.A. Ehsanpour^{1,*} and D. Twell²

¹ Department of Biology, Faculty of Sciences, Isfahan University, Isfahan, Islamic Republic of Iran

² Department of Biology, Leicester University, Leicester, UK

Abstract

SFL1 and SFL2 (SETH Four Like) genes are two members of SETH4 gene family in *Arabidopsis thaliana* expressed in saprophytic tissues. In this study, expression of SFL1 and SFL2 genes were studied using Gateway Cloning Technology. Primers were designed for PCR amplification of promoter region of SFL1 (900 bp) and SFL2 (930 bp) genes having attB1 recombination sites using Kod Hi Fi DNA polymerase enzyme. Amplified fragments were cloned into pDON201 vector by BP reaction and then into destination vector pGKWFS7 containing GFP::GUS by LR reaction. Finally, pGKWFS7-SFL1 and pGKWFS7-SFL2 were introduced into *Agrobacterium* GV3101 strain as a binary vector. Plants then were transformed with the new constructs. GUS staining of transgenic plants with SFL1 promoter showed strong expression in seedling, stem, leaf, root, root hair, sepal, silique and unevenly staining in petal. Plants transformed with SFL2 showed the same pattern of expression (except root and root hair) but relatively weaker than SFL1. No expression of either SFL1 or SFL2 was observed in pollen.

Keywords: Gateway cloning; SFL gene; GUS expression; *Arabidopsis thaliana*

Introduction

During fertilization in flowering plants, pollen is recognised by the female tissues and allowed to form the pollen tube, a tip-elongating structure delivering the sperm cells to the female gametophyte to allow fertilization. It has been estimated that at least 5,000 genes are expressed in the mature pollen grains of *Arabidopsis thaliana* [3,7,12,19]. Many of these transcripts are developmentally regulated with early and late accumulation patterns [11]. The late accumulation of pollen-specific transcripts is thought to be associated

with functions during pollen germination and tube growth. At this stage of development, the male gametophyte stores transcripts for the rapid growth occurring during the progamic phase [16].

SETH4 is an intronless gene (At4g34940) encoding a protein of 664 amino acids predicted to contain Armadillo (ARM) repeats expressed mainly in mature pollen [15]. ARM motifs are sequences of 40-42 amino acids, first identified in the *Drosophila melanogaster* segment polarity protein armadillo, the homolog of mammalian β -catenin [18]. Each ARM domain forms a 3 α -helical structure and tandem ARM repeats interact

* E-mail: ehsanpou@yahoo.com

to form a right-handed superhelix creating a site for protein-protein interaction [5,13].

The SETH4 protein showed a strong homology with two other proteins named SFL1 and SFL2 (SETH Four Like) [6]. These proteins also encoded by intronless genes (At5g66200 for SFL1 and At4g36030 for SFL2). A CLUSTAL-W alignment of the SETH4 protein with SFL1 and SFL2 highlighted the high homology between the family members. The three proteins show a strong similarity in amino acid sequence particularly in the regions corresponding to ARM domains. The next highest homology to SETH4 with 37% identity and 55% similarity is SFL3 which contains ARM repeat regions which are particularly well conserved compared with the other SETH4 family proteins. Indeed, SFL3 contains four of the putative ARM motifs corresponding to the ARM domains [6]. Since SFL3 contains most of the predicted ARM domains of the SETH4 family proteins and no other predicted protein motif, we can consider that it could be included as a member of this family. The SFL family is speculated to have arisen from an ancient duplication event. The dendrogram shows that SFL1 and SFL2 are closely related and slightly diverged from SETH4. The actual function of SFL1 and SFL2 genes has not been known [6].

Since, promoter studies have allowed the developmental regulation of a number of tissue-specific genes to be established for example, LAT52 and LAT59 promoters are coordinately activated in developing pollen grains, in close association with pollen mitosis I (PMI) [8,19], in this report, we interested in looking at the expression pattern of promoter region of SFL1 and SFL2 genes in tissues at different developmental stages of *Arabidopsis thaliana* using Gateway cloning technology to understand more details about these two genes.

Materials and Methods

Gateway Cloning

To study the expression pattern of SFL1 and SFL2 promoter in different plant organs, first, DNA was extracted from small segments of fresh leaves using CTAB extraction buffer (1.4 M NaCl, 100 mM Tris pH 8.0, 3% CTAB) followed by chloroform purification. Then, SFL1 (900 bp) and SFL2 (931 bp) promoter regions were amplified by PCR (94°, 2 min; 94°, 15 s; 45° 5 s; 72° 20 s) 25 cycle with SFL1 and SFL2 forward and reverse primers containing attB site were designed with GenJokey program and then amplification was carried out using Kod Hi Fi DNA polymerase enzyme (Novagen, 71085-3).

SFL1 primers

attB1 fw SFL1
(5'AAAAAGCAGGCTGGAGTGAGAATCCCATTG3')
attB2 rev SFL1
(5'AGAAAGCTGG GTCGTCGATATCAGATCAAATGG3')

SFL2 primers

attB1 fw SFL2
(5'AAAAAGCAGGCTCCTATTATTTGCTTGCATGTGTGT3')
attB2 rev SFL2
(5'AGAAAGCTGGGTGGATAACGAACTGTTACG3')

Amplified DNA fragments were then revealed on 1.0% agarose gel and then purified from the gel using PCR purification kit (Qiagen Gel Extraction Kit). Gateway Cloning System for BP reaction was applied according to Gateway® pDONR™ Vectors Catalog no. 11798-014, 12536-017, and 12535-035 Invitrogen, Instruction and Karimi *et al.*, 2002 [15]. Entry clones then were transformed into *E. coli* DH5a competent cells and were selected on LB medium containing 50 mg/l Kanamycin. Plasmids containing cloned SFL1 and SFL2 promoter fragments then were isolated from selected colonies. To confirm the accuracy of cloned DNA fragments pDON201-SFL1 with *Pst*I and *Hind*III and pDON201-SFL2 with *Eco*RI and *Sac*I enzymes were cut at 37° for 2 h products were then revealed on 1.0% agarose gel. In the next step LR reaction was conducted using similar condition of BP reaction. In this step SFL1 and SFL2 entry clones were transferred to destination vector pGKWS7 having GUS::GFP fusion and then cloned vectors were transferred to *E. coli* DH5a and were selected on LB medium containing Spectinomycin (100 mg/l). New plasmids named pGKWS7-SFL1prom and pGKWS7-SFL2prom were isolated from selected colonies and once again were digested with the same enzyme as mentioned before. These new constructs then were sequenced using attB1-Fw-SFL1, attB1-FW-SFL2, and GFP3'-5'CGATCACATGGTCCTGCTGG3'-primers. At the final step of cloning new constructs were isolated from *E. coli* and then were transformed to *Agrobacterium* GV1301 strain as a binary vector. Transformed GV1301 were then selected on LB medium containing gentamycin (50 mg/l), rifampicin (50 mg/l), and spectinomycin (100 mg/l).

Plant Transformation

Seeds of *Arabidopsis thaliana* wild type col-0 were planted in the mixed soil, sand, vermiculate (1/1/1) and were grown in the glasshouse at 25° under normal day light. Transformation of plants was conducted on 2-3 weeks old seedlings using method of Felmann and

Marks [10]. Inoculated plants were then grown in the glasshouse at 25° under normal day light. T1 seeds then were harvested and after disinfection with 70% ethanol were cultured on MS [17] medium containing 1% agar, 3% sucrose and 25mg/l kanamycin. T1 seedlings then were selected and grown up in the glasshouse for SFL1 and SFL2 promoter analysis.

Histochemical Analysis of GUS Expression

Plant segments in different developmental stages (13 days old seedling, early flower bud formation, mature flower, silique) were vacuum-infiltrated for 10 min in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), 0.5 M sodium phosphate pH 7.0, 0.01 M EDTA, 0.1% Triton X-100, 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide. The samples were then incubated at 37°C over night. After the stain solution was removed with 70% ethanol, the tissues were cleared and fixed in ethanol:acetic acid 3v:1v and then analysed by bright field transmitted light microscopy using an Axiophot 100 microscope (Carl Zeiss Inc.). Images were captured using a CCD camera (JVC KYF55B) and Imagegrabber software (Neotec).

PCR Testing of Transgenic Plants

For testing transformation in plants, a total genomic DNA was extracted from leaf samples according to CTAB method as described already. The attB1-fw-SFL1, attB1-fw-SFL2 and GFP3'-rev primers were used for PCR amplification of promoter fragments from SFL1 and SFL2 genes, following 94°C for 15 min, 60°C for 30 s and 72°C for 30 s. The amplified fragments were revealed on 1% agarose gel.

RT-PCR

RT-PCR was preformed for flower, leaf and stem of transgenic plants containing SFL1 and SFL2 promoter regions, using the R^{reverse-i} TTM One Step system RT-PCR Kit (AB-0845) according to manufacture's instruction to detect RNA transcripts. RNA templates from approx. 50-100 mg of fresh flower stem and leaf collected and frozen in liquid nitrogen and were then extracted with TRIZOL reagent (GIBCO BRL). RNA was also isolated from 10-20 young fresh siliques without liquid nitrogen in AMES buffer (160 ml dwater, 40 g MgCl₂, 8.21 g Na-acetate, 40 ml ethanol (96-100%), 6.0 g SDS pH 6.0). Extracted RNA was then treated with Dnase-1 enzyme to remove DNA contamination. One µl of RNA template (1 µg/ml) was mixed with 25 µl of 2× ReddyMixTM Master Mix, 1 µl primer (10 mM), 1 µl

reverse transcriptase blend and completed to 50 µl with DEPC treated water. RNA was reverse transcribed and PCR amplified using the following thermal conditions: first strand cDNA synthesis 47°C for 30 min (1 cycle), reverse transcriptase inactivation and initial denaturation 94°C for 2 min (1 cycle), denaturation 94°C for 20 s, annealing 55°C for 30 s, extension 72°C for 5 min (40 cycles) and final extension 72°C for 5 min (1 cycle).

Two-step RT-PCR conditions using Im Prom II reverse transcription kit for cDNA synthesis (Promega) with oligo dT primers were performed according to the instruction manual for increased sensitivity. The cDNA produced was diluted 40 times and 1 µl was used as a template for PCR with a reaction mixture containing 5 mM primers, 2 mM dNTPs, 1× Taq buffer and 1 unit of Taq polymerase (BIOLINE) in a final volume of 20 µl. The DNA fragment was amplified for 30 cycles using the following thermal conditions: denaturing DNA template 94°C for 30 s, primer annealing 5°C below primer T_m for 15 s, DNA synthesis 72°C for 1 min.

Primers Used for RT PCR

For SFL1

SFL1RV (5'-CTTCTCAATCAGAACAGCGAAGC-3')
SFL2FW (5'-CATCAATCGCGAAACGCGTCGG-3')

For SFL2

SFL2RV (5'-CTTTGCACGCCGGTGAAGTGCG-3')
1536DS-3 (5'-AGCCCAAGCAACAACAGTTT-3')

Results

The application of gateway cloning system in this study resulted in high efficiency cloning of promoter region from SFL1 and SFL2 genes in pGKWFS7 plasmid. Figure 1 shows PCR amplification of SFL1 and SFL2 promoter regions with *Kod Hi Fi* DNA polymerase enzyme.

Maps of the new constructs made by Gateway system for SFL1 and SFL2 promoter regions are illustrated in Figure 1 and Figure 2.

For checking the accuracy of promoter regions inserted in pGKWFS7 plasmid during cloning reactions, isolated plasmids pGKWFS7-SFL1 with *Pst*I and *Hind*III enzymes and pGKWFS7-SFL2 with *Eco*RI and *Sac*I enzymes were digested.

After pant transformation, selected plants on MS medium supplemented with kanamycin, 46 transgenic lines were tested for presence of SFL1 and SFL2 promoter regions. Figure 3 shows fragments of 1042

and 1073 bp after PCR amplification of plant genomic DNA. To evaluate the pattern of GUS expression, transgenic plants were examined with histochemical staining for GUS expression.

As complimentary experiments along with GUS staining, RT-PCR experiments carried out. As Figure 4 shows, SFL1 and SFL2 promoter regions were expressed in root, stem, leaf, flower and siliques but not in pollen of transgenic *Arabidopsis thaliana*.

GUS Staining of Plant Organs

The GUS staining pattern of different plant organs in different developmental stages, such as seedlings,

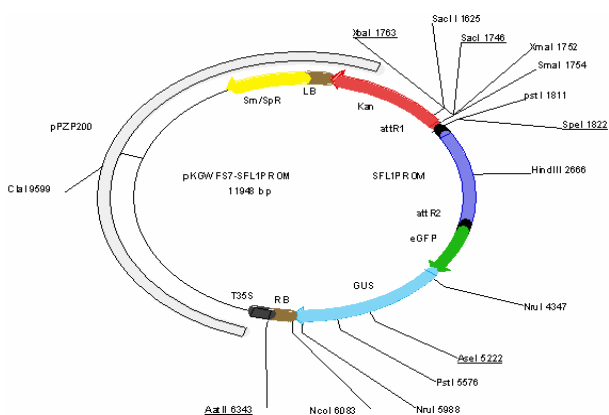


Figure 1. Map of pKGWFS7 SFL1 promoter linked to GFP::GUS for SFL1 promoter region made by Gateway cloning system.

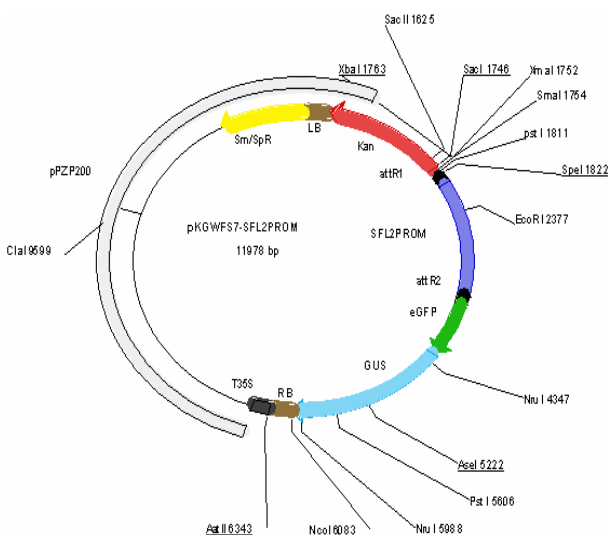


Figure 2. Map of pKGWFS7 SFL2 promoter plasmid for SFL2 promoter region with 930bp made by Gateway cloning system.

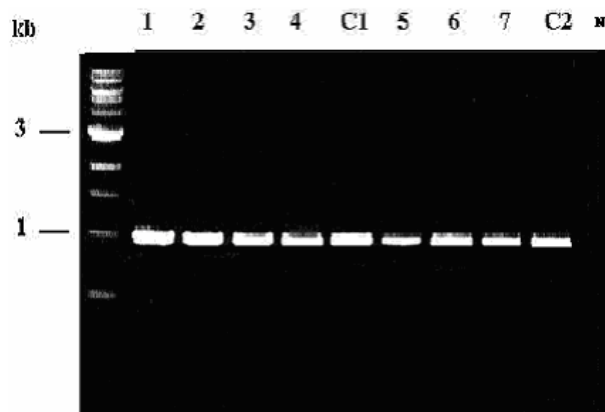


Figure 3. PCR amplification of transformed plants. Lane 1, 2, 3, 4 transgenic lines, C1 positive control for SFL1 and lane 5, 6, 7 transgenic lines, C2 positive control for SFL2 show presence of cloned DNA in transgenic plants, N: negative control.

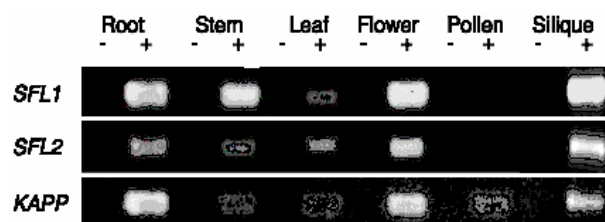


Figure 4. RT-PCR in different organs of transgenic plants. (KAPP "kinase associated protein phosphatase" used as positive control, +: with cDNA, -: without cDNA).

mature flower, silique and flower bud cluster were analyzed (Fig. 5). In 13 days stage seedling plants caring SFL1 promoter showed blue staining in cotyledon, leaf, stem, vascular tissue, root, root tip, root hair and in particular on the wall regions of silique (pattern of GUS staining was similar in siliques of transformed plants with SFL1 and SFL2 promoters). A similar pattern of GUS expression was observed on 4-5 weeks stage seedlings. Transformed plants with SFL2 promoter revealed GUS expression in leaf, stem, cotyledon, but not in root, root hair and root tip. Cluster buds of flower in both SFL1 and SFL2 promoters showed blue staining in sepal and inflorescent stem, but not at very early stage of bud development. In mature flowers GUS staining was not detected in pollen but was observed as patchy pattern in sepal and petal and evenly in inflorescent stem of transgenic plants with both SFL1 and SFL2 promoters. As a general point blue staining in tissues with SFL2 promoter were slightly weaker than SFL1.

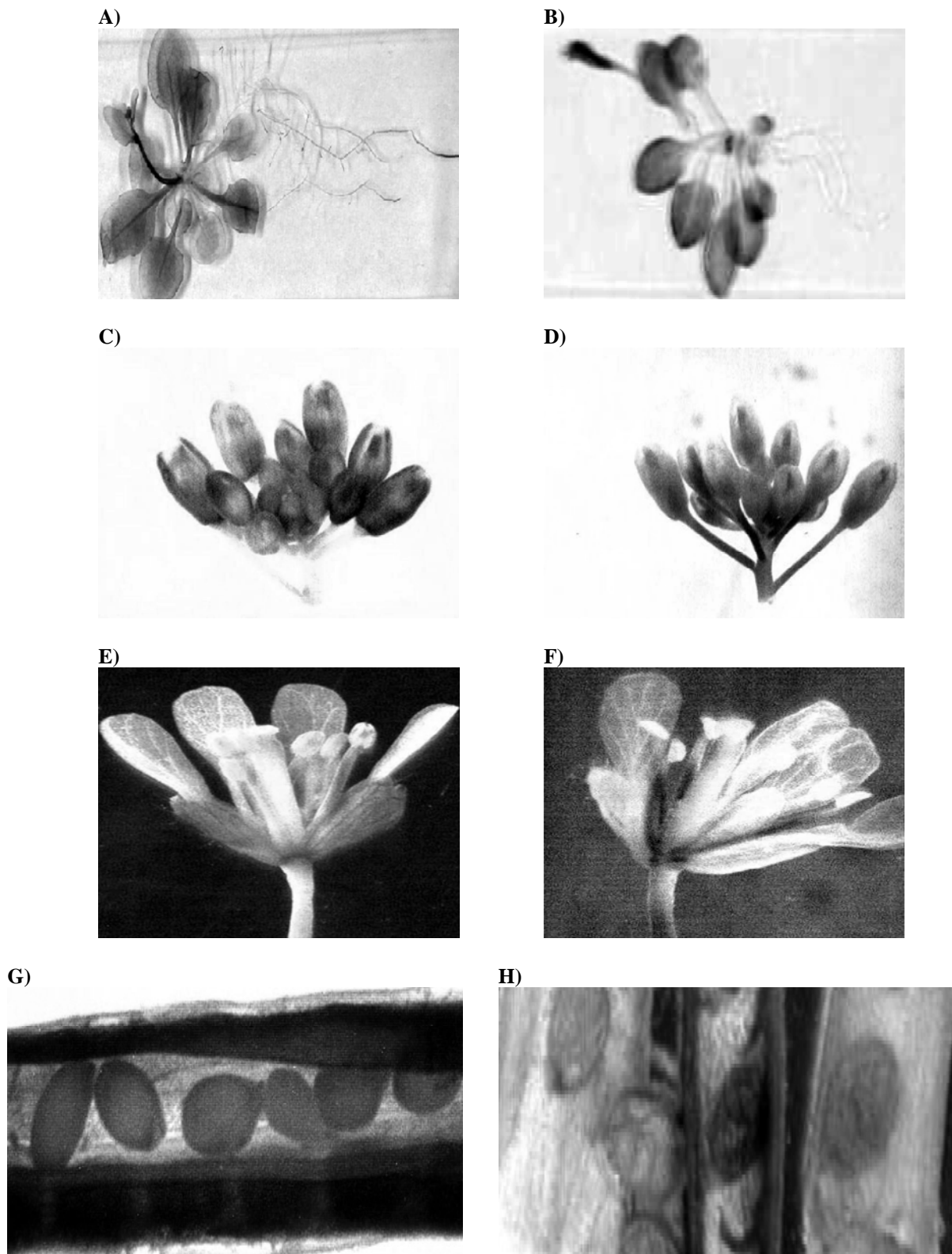


Figure 5. Expression pattern of GUS gene in transgenic plant containing SFL1 promoter region (A, C, E, G) and SFL2 promoter region (B, D, F, H). A and B: 13 days stage seedlings; C and D: 4 weeks stage flower cluster buds; E and F: 6 weeks stage mature flowers; G and H: young siliques.

Discussion

Numbers of studies provide evidence for a developmental switch in transcriptional activity at different stages. For example, promoter mutagenesis studies have demonstrated that discrete regions of the promoter are active at different times during maturation of plant organs [2]. This provides a composite structure that has evolved to allow rapid activation of promoter in specific tissue and maintain high levels throughout maturation. With regard to the organization of regulatory elements, which has been defined for a number of tissue-specific promoters, some general features have emerged. First, cis-regulatory elements sufficient to direct transcription in cells are always located relatively close to the transcription start site. Second, multiple upstream promoter elements increase transcriptional activity of tissue-specific core promoter regions. Some of these elements are insufficient to activate tissue expression, whilst others also possess specific enhancer activity, and are able to activate promoter sequences. These suggesting a positive regulatory mechanism, involving the tissue-specific expression or activation of transcription factors [9].

It has been known that having multiple genes encoding similar proteins under the control of different expression signals enables the plant to respond with increased flexibility to developmental and environmental signals. In addition to that, non identical but very similar proteins that are expressed simultaneously in cells could allow for greater functionality and stability of the processes of the proteins is involved. In our study, SFL1 and SFL2 promoters are expressed at early stage of seedling development. Expression pattern of GUS gene linked to SFL1 and SFL2 promoter regions indicated that in seedling cotyledon, stem, leaf, root and in particular root tips which are growing and expanding tissues showing blue staining. In contrast, evaluation of SETH4 promoter region in *Arabidopsis thaliana* has shown that no GUS expression can be revealed in seedling [6]. At the early stage of flowering (cluster bud) and mature flowers tissues which are growing and expanding such as sepal and petal or inflorescent stem and siliques, GUS gene is strongly expressed. At this stage we do not know exactly why the expression of SFL2 is relatively weaker than SFL1 promoter. Our investigation showed that, this phenomenon is not related to the cloning system but rather could be a weaker roll of SFL2 protein in the cells. Our data confirmed that pattern of GUS expression of SETH4 promoter region in flowers of *Arabidopsis thaliana* reported by Christos [6] are more and less similar to SFL1 and SFL2 promoter region

except pollen. Expression of SETH4 promoter is mainly localized in pollen while SFL1 and SFL2 promoter are never expressed in pollen. It seems that SFL1 and SFL2 proteins containing ARM repeats may implicate in cell signalling, localising proteins to organelles and may acting as transcription regulators. The localization pattern of GUS expression for the SFL1, SFL1 and SETH4 promoters [6] indicating that possible interaction with another protein/signaling molecule or possibly the transient localisations of the proteins to the cell wall as an expanding site [1]. Another explanation of this pattern of expression is activity of actin filaments in expanding sites. For example, the regulation of pollen tube growth is known to involve alterations in intracellular calcium levels, phosphoinositide signalling, and signalling pathways and vesicle transferring to the tip which operate through the actin cytoskeleton [4]. The Rho small GTP-binding proteins are versatile, conserved molecular switches in eukaryotic signal transduction. Plants contain a unique subfamily of Rho-GTPases called Rop (Rho-related GTPases from plants). For example, pea Rop1Ps is predominantly expressed in pollen and localised towards the apex and at the periphery of the generative cell [14]. This suggests that Rop1Ps may modulate tip growth however; SFL1 and SFL2 may have a similar roll in regulation of actin assembly via GTPases. However, it is clear that SFL1 and SFL2 genes are expressed in different saprophytic tissues of *Arabidopsis thaliana* under control of developmental cell signaling.

Acknowledgment

The authors wish to thank head of research Committee Prof. Mohamad Ali Shahzamanian and members of Isfahan University Research Committee for their support during sabbatical at Leicester University in UK.

References

1. Amador V., Monte E., García-Martínez J.L., Prat S. Gibberellins signal nuclear import of PHOR1, a photoperiod-responsive protein with homology to *Drosophila armadillo*. *Cell* **106**: 343-354 (2001).
2. Bate N. and Twell D. Functional architecture of a late pollen promoter: pollen specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. *Plant Mol. Biol.*, **37**: 859-69 (1998).
3. Becker J.D., Boavida L.C., Carneiro J., Haury M., Feijo J.A. Transcriptional profiling of *Arabidopsis* tissues reveals the unique characteristics of the pollen transcriptome. *Plant Physiol.* **133**: 713-725 (2003).
4. Clarke S.R., Staiger C.J., Gibbon B.C. and Franklin-Tong V.E. A potential signaling role for profilin in pollen of

- Papaver rhoeas. *Plant Cell*, **10**: 967-79 (1998).
5. Conti E. Kuriyan J. Crystallographic analysis of the specific yet versatile recognition of distinct nuclear localization signals by karyopherin α . *Structure* **8**: 329-338 (2000).
 6. Christos M. (2004) Characterisation of gametophytic mutants affecting pollen function in *Arabidopsis thaliana*. PhD Thesis, Leicester University, Leicester, UK.
 7. Da Costa-Nunes J.A., Grossniklaus U. Unveiling the gene-expression profile of pollen. *Genome Biol.* **5**: 205 (2003).
 8. Eady C., Lindsey K. and Twell D. The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. *Plant Cell*, **7**: 65-74 (1995).
 9. Eyal Y., Curie C. and McCormick S. Pollen specificity elements reside in 30 bp of the proximal promoters of two pollen-expressed genes. *Plant Cell*, **7**, 373-84 (1995).
 10. Felmann K.A. and Marks M.D. Agrobacterium-mediated transformation of germinating seeds of *Arabidopsis thaliana* using a non-tissue culture approach. *Mol. Gen. Genet.* **208**: 1-9 (1987).
 11. Honys D., Combe J.P., Twell D. and Capková V. The translationally repressed pollen-specific ntp303 mRNA is stored in non-polysomal mRNPs during pollen maturation. *Sex Plant Reprod.*, **13**:135-44 (2000).
 12. Honys D. Twell D. Comparative analysis of the Arabidopsis pollen transcriptome. *Plant Physiol.* **132**: 640-652 (2003).
 13. Huber A.H., Nelson W.J., and Weis W.I. Three-Dimensional Structure of the Armadillo Repeat Region of β -Catenin. *Cell* **90**: 871-882 (1997).
 14. Lin Y., Wang Y., Zhu J. and Yang Z. Localization of a Rho GTPase implies a role in tip growth and movement of the generative cell in pollen tubes. *Plant Cell*, **8**: 293-303 (1996).
 15. Karimi M., Inze D. and Depicker A. Gateway vectors Agrobacterium mediated plant transformation. *Trends in Plant Science* **7**: 193-195 (2002).
 16. McCormick S. Control of male gametophyte development. *Plant Cell* **16**:S142-S153 (2004).
 17. Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant* **159**: 473-479.
 18. Riggleman B., Wieschaus E. and chedl P. Molecular analysis of the armadillo locus: uniformly distributed transcripts and a protein with novel internal repeats are associated with a Drosophila segment polarity gene. *Genes Dev.* **3**: 96-113 (1989).
 19. Twell D., Yamaguchi J. and McCormick S. Pollen-specific expression in transgenic plants: coordinate regulation of two different promoters during microsporogenesis. *Development*, **109**: 705-713 (1990).