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# Isolation and cloning of fruit-specific *E8* promoter from local small fruit tomato (*Lycopersicon esculentum* L.) and analysis its transient expression

**Davood Yavari[1], Seyyed Abolghasem Mohammadi[1,*], Ebrahim Dorani Uliae[1]**

[1]*Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran*

**Key words:** *E8*, reporter gene, specific promoter, tomato, transient expression.

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**Abstract**

Isolation and characterization of specific promoter is important for efficient expression of recombinant proteins in plants. *E8* is a fruit specific promoter with 2.2 kb length. To isolate *E8* fruit specific promoter, seeds of local small fruit Sardasht genotype were sown in greenhouse and genomic DNA was extracted from young leaves using CTAB method. The promoter region was amplified using specific primers designed based on conserved regions of the sequences available at NCBI database. The amplified fragments ligated into PTZ57R/T vector and transferred into competence cells of DH5α strain of *E. coli* for cloning. Plasmid was extracted after confirming the presence of the inserted segments in plasmid based on colony PCR test and enzyme digestion. The extracted plasmids were used for sequencing the fragments. Sequence analysis revealed successful isolation of *E8* promoter segment. To analyze the activity promoter in tomato tissues, the *CaMV*35S promoter in pBI121 binary vector replaced by *E8* promoter and transferred to GV3101 strain of *Agrobacterium*. Transient expression was assessed using *gus* gene under *E8* promoter with aim of transgenic *Agrobacterium* agro-infiltrated into fruit tissue. Presence of indigo color indicated successful expression of *gus* reporter gene under *E8* promoter at the cells receipt the gene construct.

* **Corresponding Author:** Seyyed Abolghasem Mohammadi ✉ mohammadi@tabrizu.ac.ir

## Introduction

Plant-produced proteins are considered to be safe, as plants do not harbor human pathogens such as human immunodeficiency virus, hepatitis viruses, and toxins which animal/animal cell culture systems often do (Larrick and Thomas, 2001). Several factors including codon usage, stable and efficient expression of foreign gene, need to be considered in order to maximize the yield of recombinant proteins in transgenic plants, Although, procedure, timing, and localization of gene expression are regulated by a hierarchy of control mechanisms, promoter, as one of main transcript regulators, plays a significant role in gene expression. The Cauliflower mosaic virus 35S (*CaMV*35S) promoter is widely use to drive foreign gene expression in plant cells (Jani *et al*., 2002). However, this promoter does not confer any specificity-neither tissue specificity nor plant developmental stage specificity on exogenous gene expression leading to lower expression levels (Smigocki and Owens, 1988). In production of vaccines in transgenic plant, low expression level will lead to the decrease of immunogenicity, which will trigger insufficient protection or immunological tolerance to human body. A major advantage of targeting protein expression to fruits is that the edible parts can be consumed uncooked or partially processed, making them convenient sites for the production of vaccines.

Several fruit-specific promoters such as *E4*, *E8*, *PG* and *2A11* have been identified in tomato (Coupe and Deikman, 1997; Deikman *et al*., 1998). These promoters have been mostly used to investigate the role of ethylene in fruit ripening. The *E8* promoter is one of the most extensively characterized ripening-specific tomato promoters. It was found that *E8* promoter has at least two main regions contributing its transcriptional regulation; the region of –2181 to –1088 containing DNA sequence that confer ethylene responsiveness in unripe fruit but are sufficient for *E8* gene expression during ripening, and the 'downstream' region of –1088 to the transcriptional start site is sufficient for ripening-specific transcription in the absence of ethylene synthesis (Deikman *et al*., 1992). Some researchers used the *E8* promoter to drive the expression of exogenous genes in transgenic tomato fruits (Krasnyanski *et al*., 2001; Mehta *et al*., 2002; Yakoby *et al*., 2006), however, there is no report on the gene expression driven by the *E8* promoter in the small fruit tomato as far as we know.

The purposes of this study were to i) isolate and sequence *E8* promoter from local small fruit Sardasht genotype and ii) compare its tissue-specific expression in tomato fruits with *CaMV*35S promoter using transient expression agro-infiltration method.

## Materials and methods

*Plant material and DNA isolation*

A local small fruit tomato (Sardasht) genotype was used in this experiment. Seeds were sown in greenhouse for DNA extraction. Young leaves at the 8–10 leaf stage were collected, and genomic DNA was extracted by the cetyltrimethyl ammonium bromide (CTAB) method (Saghai-Maroof *et al*., 1984). Final concentration of total DNA was adjusted to 50 ng/µl.

*Isolation and cloning of promoter region*

*E8* promoter was amplified using specific primers designed based on the conserved region of *E8* sequence from NCBI database. The forward and reverse primer sequences were 5′-aagcttctagaaatttcacgaaat-3′ and 5′-tctagacttcttttgcactggaatga-3′, respectively. The PCR program was initiated with a hot start at 94°C for 4 min, and followed by 35 cycles (94°C for 30 s, 51°C for 50 s, 72°C for 90 s) and finally 72°C for 7 min.

The target PCR products were recovered with DNA Purification Kit (silica Beed DNA Extraction Kit-#K0513) and subcloned into pTZ57R/T (InsTAcloneTM PCR Cloning Kit# K1214, Fermentas). The recombinant plasmid was transformed into the *E. coli* strain DH5α by using a freeze-thaw method (Sambrook and Russell, 2001). Transformed *E. coli* DH5α was grown on LB agar plates containing ampicillin (100µg/ml) and positive clones were selected by colony PCR, plasmid PCR and

then were identified by double enzyme digestion of *Hind*III and *Xba*I. The positive recombinants were sequenced. Sequences of *E8* promoter and X13437.1 were aligned by online bl2seq program (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). Cis-elements of *E8* promoter were analyzed with online analysis tools PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo *et al*., 1999).

*Construction of binary vector*

The positive recombinant pTZ57R/T-E8 and empty vector pBI121 were digested by *Hind*III and *Xba*I. The small digestion fragment of recombinant pTZ57R/T-E8 and the large fragment digestion product of pBI121 were recovered, respectively. The purified products were ligated overnight and ligated products were transformed into *E. coli* DH5α competent cells and cultured on LB medium with 50 μg/ml kanamycin. Plant expression vector pBI121-E8 was identified by colony PCR, and double enzyme digestion of *Hind*III and *Xba*I. The pBI121-*E8* and pBI121 as a control were transformed into *Agrobacterium tumefaciens* strain GV3101 through Freeze-Thaw method (Sambrook & Russell, 2001) and confirmed by colony PCR and was used in a transient promoter assay.

*Transient expression assay*

*Agrobacterium tumefaciens* strain GV3101 was inoculated into 50ml LB medium supplemented with 50 mg/l of kanamycin and rifampicin grown overnight to logarithmic phase (OD600= 0.6) at 28°C. Bacteria were centrifuged and resuspended in half volume of infection medium (Murashige and Skoog, (1962): basal medium (pH 5.5) containing 5.0% sucrose, 10 mM MES and 200 mM acetosyringone) and grew at 28°C for 2-3 h to a final OD600= 0.6. Slice tomatoes were added to the solution and vacuum for15 minutes. After infiltration, vacuum was broken rapidly. Slice tomatoes were rinsed in sterile water, kept on a Whatman paper # 40 with adaxial side facing up and put in sealed trays (16/8 h photoperiod, 25°C) for 72h. Histochemical reactions with the indigogenic substrate, X-Gluc were performed with 1 mM substrate in 50mM $NaH_2PO_4$, pH 7.0 at 37°C for times from 20 min to several hours. After staining, sections were rinsed in 70% ethanol for 5 min (Jefferson *et al*., 1987).

**Results and discussion**

We successfully amplified a 1.1 kb fragment of *E8* gene promoter which was consistent with the predicted length (Figure 1).

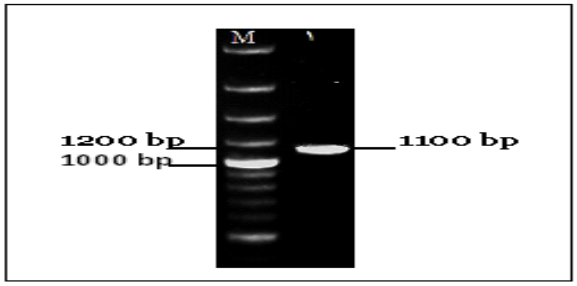


**Fig. 1.** Agarose gel separation of PCR product of *E8* promoter. Note: M-DNA Molecular weight marker 3000 bp ladder; 1: PCR product of *E8* promoter.

The target PCR products were ligated to pTZ57R/T and the ligated products were transformed into competent cells of *E. coli* DH5α. The transformants were firstly selected by colony PCR and then were identified by double enzyme digestion with *Hind*III and *Xba*I. The digest products of the positive recombinants, 1100 bp (Figure 2), were consistent with the predicted length, which indicated that they were suitable to be sequenced.

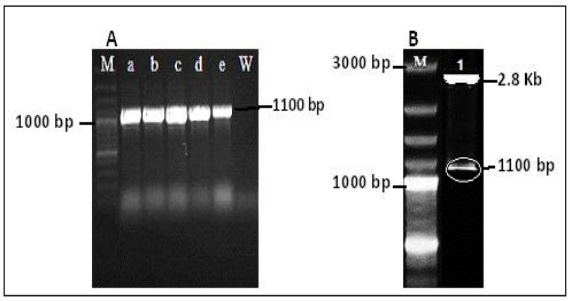


**Fig. 2.** Molecular analysis of *E8* promoter in pTZ57R/T cloning vector. A: PCR analysis of bacterial colony containing the product of pTZ57R/T+ *E8* ligation for the presence of 1100 bp*E8* promoter. B: Enzymatic digestion of the recombinant plasmid using *Hind*III and *Xba*I. W-Negative control (water); M-DNA molecular weight marker 3000 bp ladder; 1: Recombinants digested by *Hind*III and *Xba*I; a~e: Different colony containing *E8* promoter.

The sequencing results of the two recombinants of *E8* promoter were the same and the length was 1113bp (Figure 3). Online BLAST analysis revealed 98.33% similarity between cloned *E8* promoter and other *E8* promoter sequences from NCBI(X13437, DQ317599 and AF515784). Whereas, sequence similarity between our sequence and *ACO* gene mRNA and *E8* gene cDNA was 38 %. Identification and verification cis-acting elements in *E8* promoter were carried out by Online PLACE analysis database. Through alignment of the *E8* promoter, the consensus sequences of promoters in eukaryotic genes such as TATA box, CAAT box1 and CCAAT box were detected in the sequenced E8 promoter (Shirsat*et al.*,1989; Grace *et al.*, 2004). The number of TATA box, CAAT box and CCAAT box were 15, 9 and 4, respectively. The 11 TATA boxes include 3 TATA box2 (TATAAAT), 2 TATA box3 (TATTAAT), 9 TATA box5 (TTATTT) and 1 TATABOXOSPAL (TATTTAA). Some cis-elements, which were related to development and ripening of fruit, were also found in the *E8* promoters. The ethylene-responsive element (ERE) is related to ethylene response, and the consensus sequence of the ERE is an 8 bp ATTTCAAA motif (Itzhaki *et al.*,1994; Tapia *et al.*,2005).The *E8* promoter includes two copies of the ERE motif which were located from -834 to -841 and -991 to -997, respectively. An 8 bp motif of TAAAATAT is the Cysprotease-binding site in tomato, and plays an important role in ethylene biosynthesis of tomato (Matarasso *et al.*, 2005). Two copies of the TAAAATAT motif were also indentified in *E8* promoter and they were located from –430 to –437 and –908 to –901, respectively. The 6 bp TGTCTC motif is an auxin response factor (ARF)-binding site, which was found in the promoters of the primary/early auxin response gene of Arabidopsis thaliana (Hagen and Guilfoyle., 2002; Nemhauser *et al.*, 2004). The TGTCTC motif was located from –960 to –965 in the *E8* promoter. The distal portion of B-box (dist B) element is responsible for seed-specific expression and abscisic acid (ABA) response in the napA gene of *Brassica napus* (Ezcurra *et al.*, 2000). The consensus sequence of dist B is GCCACTTGTC, and located from –609 to –618 in the *E8* promoter.

The transient expression of *gus* gene in tomato tissue was evaluated using Agro-infiltration method. Transient expression through agro-infiltration is a relatively simple procedure. The most time consuming step is cloning a transgene construct under the control of a tissue-active or constitutive promoter into a binary vector. Agro-infiltration is simple and effective, involving the injection of *Agrobacterium tumefaciens* into leaves or organs of interest, and then monitoring transient transgene expression within the infiltrated tissue during the next few days (Sparkes *et al.*, 2006). Agro-infiltration has been demonstrated to be effective for transient expression in many plant species including tobacco (Sheludko *et al.*, 2006), lettuce, tomato, Arabidopsis (Wroblewski *et al.*, 2005), radish, pea, lupine, and flax (Van der Hoorn *et al.*, 2000).

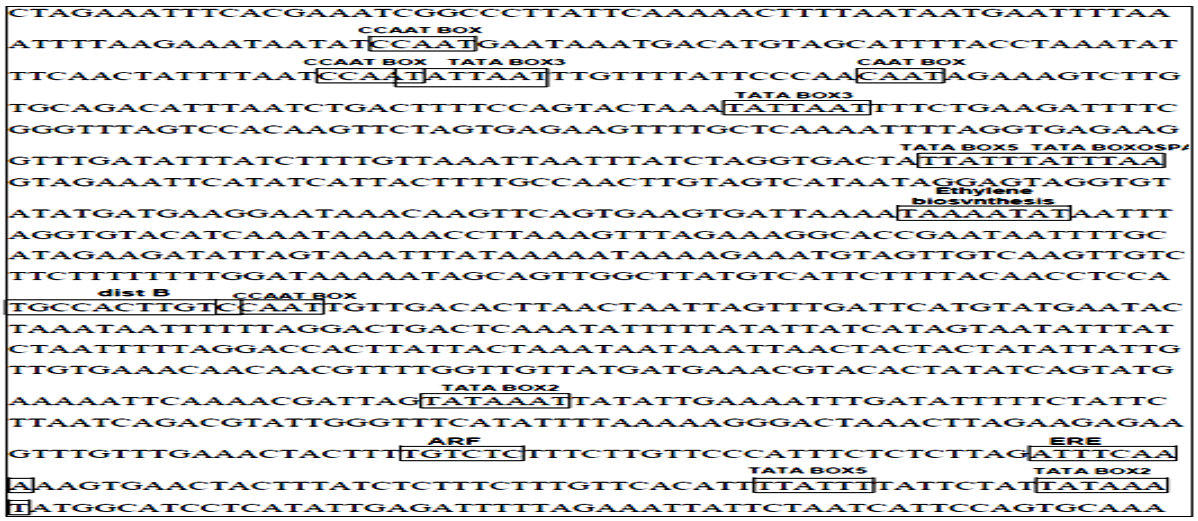


**Fig. 3.** Sequence of *E8* promoter with detected important cis-elements**.**

Results from transient expression experiment of tomato fruit in X-Glu solution indicated that *E8* and *CaMV*35S promoters both led to *gus* gene expression in tomato fruit. This matter in addition to show activity and efficiency of *E8* promoter also implies succession of Agro-infiteration method in tomato fruit. Indigo color of tissues showed that the *gus* gene expression done by promoter. Control samples with no color emphasis the accuracy of gene expression and ineffectuality of lateral and environmental results (Figure 6).

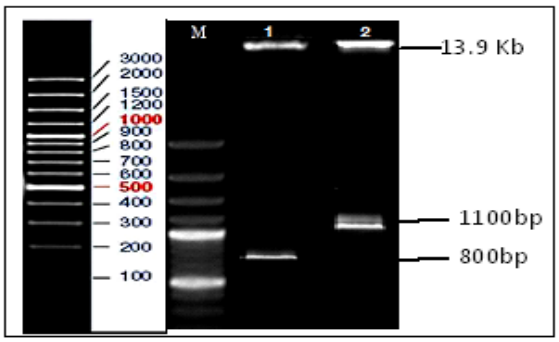


**Fig. 4.** Enzymatic digestion of pBI121-E8. M-DNA molecular weight marker 3000 bp ladder; 1,2-Respectively pBI121 and Recombinant pBI121-E8 digested by *Hind*III and *Xba*I.

Transient gene expression of Organophosphorus pesticide (opd) degradation under *E8* promoter control in tomato plant with Agro-filteration method led to its specific expression in fruits of this plant (Zhao and Zhao, 2009). In this research also researchers could able to expression of *gus* gene with the same method under *CaMV*35S promoter in tomato fruit. Orzaez *et al.*,(2006) in a research developed transient expression for gene function analysis in tomato fruit and showed that injection of Agrobacterium suspension by Agro-injection led to more bacteria penetration in fruit. Stable *gus* gene expression under 2/2 bp of *E8* promoter in tomato fruit indicated that *gus* gene expression in immature fruits occurs only in vascular paranshim cells while in ripen fruits occurs in whole fruit (Kniessl and Deikma, 1996). In present study we used 1/1 bp of *E8* promoter segment instead of its 2/2bp. The results indicated that type of promoter segment also can induce *gus* gene expression in ripen fruits. Zhou *et al.*, (2003) in a research could cloning as well as sequencing of *E8* promoter. They showed that it is possible to use this promoter in edible vaccine production in transgenic tomato plants. He *et al.*, (2007) analysis the tomato specific *E8* promoter in vaccine antigen expression. They isolate 1/1 kb and 2/2 kb promoters from *Lycopersicon esculentum* and sequencing them. They bound the *E8* and *CaMV*35S promoters to *HBsAg* gene and transferred to *Nicotiana tabacum*. *HBsAg* expression under 1/1 kb promoter control did not observe in leaf tissue, flower and seed. However under *CaMV*35S promoter control expression observed in transgenic tobacco. With use of ELISA assessment, it was proved that 1/1 kb segment of *E8* promoter could able to expression of extrinsic gene in ripen transgenic tomato fruits while less expression found in leaves, flowers and immature fruits. Results showed that this promoter is practicable not only in specific organs but also in specific species. Jiang *et al.*, (2007) reported that 1/1 kb segment of promoter could lead to efficiency gene expression in tomato fruit. It is seemed that other parts of 2/2 kb promoter may be increased gene expression (Deikman *et al.*, 1998). Therefor we can use this promoter for efficiency production of protein and recombinant vaccine in tomato fruit.

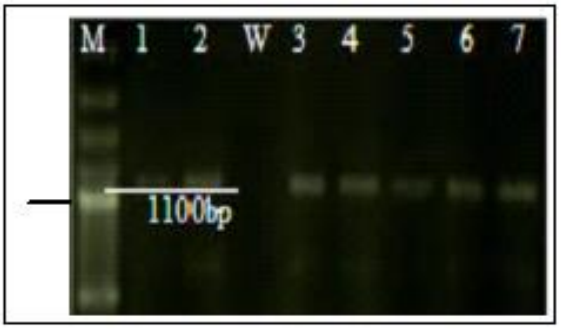


**Fig. 5.** PCR analysis of *Agrobacterium tumefaciencs* GV3101 containing pBI121-E8 recombinant vector. M-DNA Molecular weight marker 3000 bp ladder; 1~7: Different agrobacterial colony containing pBI121-E8; W-Negative control (water).

In recent years, plant has emerged as a convenient, safe and economical alternative to the mainstream expression system for pharmaceutical protein production including antibodies, vaccines, industrial enzymes, biopolymers and so on (Schillberg *et al.*, 2005). Two species of plant, tobacco and tomato, hold

their own advantages on this research area. Tomato is widely used for plant vaccines due to the feature of its fruit being palatable, nutritionally attractive, and could be eaten fresh. The activity and specificity of a promoter, the main transcriptional regulator, can affect the level of transgene expression in plant. The *E8* promoter has the fruit specific expression feature, so it would be propitious to the growth of transgenic plant, harvest of the fruits, and the use of transgenic plant-made vaccine.

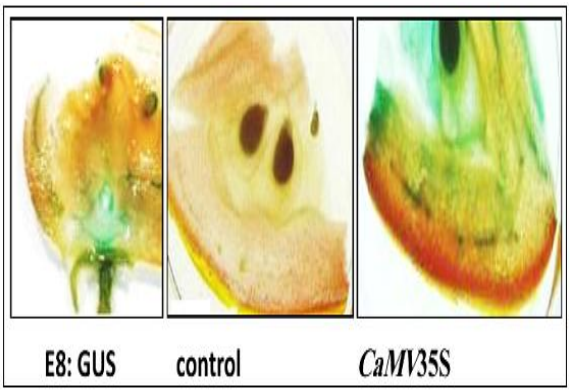


**Fig. 6.** Transient expression of gus gene under the control of E8 and *CaMV*35S promoter using Agroinfilteration system. Control-agroinfilteration using*agrobacterium* free from plasmids.

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