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# Isolation and characterization of Ethylene Responsive Factor (ERF) genes from brinjal

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## ABSTRACT

**Ethylene Responsive Factor (ERF) family of genes encode transcriptional regulators with a variety of functions involved in the developmental and physiological processes in plants. In this study, six *SmERF* genes were isolated from brinjal and expression analysis was carried out to determine their role in response to salt, drought and mechanical wounding and during fruit development and ripening stages. All the six *SmERFs* displayed differential expression pattern and levels throughout various stages of fruit development and ripening. Six genes namely *SmERF7*, *SmERF20*, *SmERF52*, *SmERF70*, *SmERF80* and *SmERF83* were up-regulated in response to salt, drought and mechanical wounding suggesting a crosstalk between stress signaling pathways. Up-regulation of *SmERF7*, *SmERF52*, *SmERF70* and *SmERF83* play important role at early stages of fruit development.**

**Key words:** Ethylene Responsive Factor, brinjal, fruit ripening, stress response.

## INTRODUCTION

Brinjal (*Solanum melongena* L.) commonly known as eggplant is an agronomically important crop, grown primarily for its fruits. This is the third important Solanaceae vegetable after potato and tomato. It is native to India and probably introduced to Europe by Arabian traders and then taken to North America by early European settlers. Most of the commercially important varieties of brinjal have been selected from the long established types of the tropical India and China. Brinjal has many unique traits, including large fruit size, high temperature and water-stress tolerance, *Verticillium* and bacterial wilt resistance etc. (Saito *et al*., 12). Fleshy fruits are divided into two groups, climacteric and non-climacteric, based upon the presence or absence of steep increase in respiratory rate and ethylene evolution, brinjal is a non-climacteric fruit. It does not have an autocatalytic ethylene burst during ripening and exogenous application of ethylene does not rapidly accelerate fruit ripening. It is interesting to study the behavior of Ethylene Responsive Factor (ERF) genes in this species to understand difference between climacteric and non-climacteric fruit ripening.

The ERF family is a large gene family of transcription factors and is a part of AP2/ERF superfamily (Riechmann *et al*., 11). The AP2/ERF superfamily is defined by the AP2/ERF domain, which consists of about 60 to 70 amino acids and is involved in DNA binding. AP2/ERF proteins have important functions in the transcriptional regulation of a variety of biological processes related to growth and development, as well as various responses to environmental stimuli. *Apetala* 2 family genes have been shown to participate in the regulation of flower and embryo development (Boutilier *et al*., 2). Many proteins of ERF family were identified and implicated in many diverse functions in cellular processes, such as response to biotic (Gu *et al*., 6) and abiotic stresses (Dubouzet *et al*., 4), regulation of metabolism (Aharoni *et al*., 1; Zhang *et al*., 15) and in developmental processes (Chuck *et al*., 3). The objective of this study was to isolate and characterize the expression of selected members of brinjal ERF gene family under stress conditions and during fruit development and ripening stages.

## MATERIALS AND METHODS

All the six genes encoding Ethylene Response Factor (ERF) were identified by homology search using ERF genes from tomato (Sharma *et al*., 13). Brinjal ERF gene ESTs were searched using BLAST, at eggplant gene index project database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=eggplant), taking tomato ERF ESTs as the reference. Primers were designed to amplify selected genes from brinjal cDNA isolated from the seedling tissues (Table 1). For gene amplification, polymerase chain reaction (PCR) was performed in a final volume

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**Table 1.** List of the primers used to amplify *SmERF* genes from brinjal.

| Primer code | Sequence |
|---|---|
| ERF 7 | F: CGAGGAGTGAGACAACGCCATTGGG<br>R: GCAGGAACA AGATTGGAGAGTGACC |
| ERF 20 | F: CCGCGATAGCAGCAAGCATCCTG<br>R: CTCCATCTTCCGGATATAGCCAGGC |
| ERF 52 | F: CGTGGCGTCCGTCAGCGACATTGG<br>R: CTGTTGTGTACTCTCTGCTTG ACC |
| ERF 70 | F: CCACCGATGAACTTTCCGGGAGA<br>R: CCGACGAGCTAGTAGCCA GTTCAG G |
| ERF 80 | F: GGGCAGCTAGAGTATGGCTCGG<br>R: CCC TCA GCT TTC TCA GGC CCC CAC |
| ERF 83 | F: GGGCAGCAGAAATAAGGGATCCACG<br>R: ATATCTGCCCAATAGTCTCTCGCC |

of 25 µl containing 10 × buffer, 2.5 mM dNTPs, 1 unit of *Taq* DNA polymerase, 10 pmols/reaction primer and 50 ng of cDNA. The PCR was performed by initial denaturation at 94°C for 5 min. followed by 35 cycles of denaturation at 94°C for one min., annealing at 62°C for 45 s, extension at 72°C for two min. and final elongation at 72°C for 7 min. Amplified products were confirmed by Sanger sequencing from both the sides using forward and reverse primers. Confirmed six gene sequences of *SmERF7*, *SmERF20*, *SmERF52*, *SmERF70*, *SmERF80* and *SmERF83* were submitted to NCBI.

Seeds of brinjal (*Solanum melongena* L.) cultivar Pusa Uttam were germinated on Murashige and Skoog (MS) medium and 15-17 day-old seedlings were subjected to three different stresses, *i.e.* mechanical wounding, salt and drought stresses. For salt and drought stresses seedlings were removed from the solid MS medium without disturbing roots and transferred to liquid MS medium containing 200 mM NaCl and 200 mM mannitol, respectively. For mechanical wounding, each leaf and cotyledon of the plant was punctured three times with a needle and squeezed twice with forceps. All the stressed samples were collected after 8 h of stress. For different fruit developmental stages, brinjal plants were grown under controlled conditions in the glasshouse. Fruit samples were collected at 0, 5, 10, 20 and 50 days post anthesis (dpa). All the samples were frozen in liquid nitrogen just after collection and stored at -70°C.

Total RNA was isolated using Spectrum™ Plant Total RNA kit (Sigma, USA) according to the manufacturer's protocol. The quality and quantity of RNA was measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) and gel electrophoresis. First strand cDNA was synthesized from 1 µg of total RNA using AffinityScript qPCR cDNA synthesis kit (Stratagene, Agilent Technologies, USA). Gene specific qRT-PCR primers were designed using PrimerQuest software (http://eu.idtdna.com). The primer sequences are given in Table 2. qRT-PCR was performed using the Brilliant-II SYBR Green qPCR master mix in Stratagene MX3005P (Agilent Technologies, USA) detection system. The qRT-PCR was performed using following PCR conditions: DNA denaturation for 5 min. at 95°C, followed by 40 cycles of amplification consisting of 30 s of denaturation at 95°C, 30 s of annealing at 60°C and 30 s of extension at 72°C. Three biological replicates were used in this experiment. Amplicons were subjected to the meltcurve analysis to check the specificity of the amplified products. The relative expression level of each gene was calculated by $2^{-ddCt}$ and 18S rRNA gene was used as housekeeping gene to normalize the amount of template cDNA added in each reaction.

## RESULTS AND DISCUSSION

In the present study six *ERF* genes from brinjal (*SmERF7*, *SmERF20*, *SmERF52*, *SmERF70*, *SmERF80*, *SmERF83*) were isolated and amplified from cDNA (Fig. 1). PCR products were further sequenced from both the sides and submitted to NCBI (Accession No. KF547974- KF547979). The expression analysis was carried out for these *SmERFs* after 8 h of salt, drought and mechanical wounding stresses and during fruit development (5, 10, 20 dpa) and ripening

**Table 2.** List of *SmERF* primers used in qRT-PCR analysis.

| Primer code | Sequence |
|---|---|
| SmERF7 | F: GCCAAAGCTTGGCCAATCCCAAAT<br>R: AGCTGAACCTGAATCCACTGAAGC |
| SmERF20 | F: GCAGCAGCAATGGACAAGTTCGAT<br>R: CGATGTTGCTAAGTCTATCGCGGA |
| SmERF52 | F: TCGACGAAGTATCTCTCTGCTGCT<br>R: CTTGCAATTTCGTCGTTCCAGGTC |
| SmERF70 | F: CGGATTGGTTTGAATGAACCGGAACC<br>R: CAACGTCACATTTCCGAACGGCTT |
| SmERF80 | F: CCACAATGCTTCGTCTTCTTCACC<br>R: TGTCCGATGCCATCAATTCATCC |
| SmERF83 | F: GCATCTCCGCAATTCATAGCTCCA<br>R: GTGAGTATTCCCAATAGTCTCTCGCC |
| 18S rRNA | F: CCGCGGAAGTTTGAGGCAATAACA<br>R: CGGCAAGGCTATAAGCTCGTTGAA |

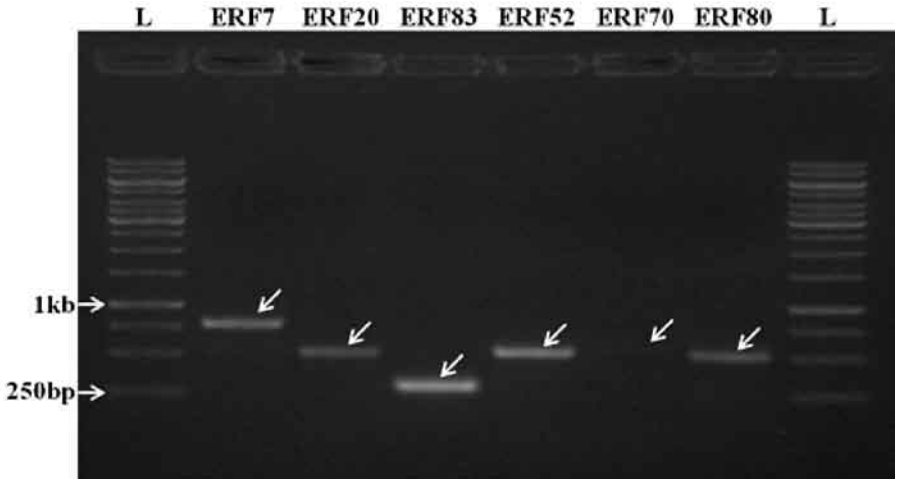


**Fig. 1.** PCR amplification of partial *SmERF* genes from brinjal seedling cDNA. L: 1kb DNA ladder.

(50 dpa) stages. All the analyzed genes exhibited differential accumulation in response to three given stress treatments (Fig. 2). For *SmERF7*, *SmERF70*, *SmERF80* and *SmERF83* genes magnitude of the fold change under salt stress was more compared to drought and wound stress, while expression of *SmERF20* and *SmERF52* was more during salt stress. In wounding stress, all *SmERF* were up-regulated with fold change varying from 8.1 (*SmERF83*) to 47 fold (*SmERF80*) except in *SmERF7*, where no

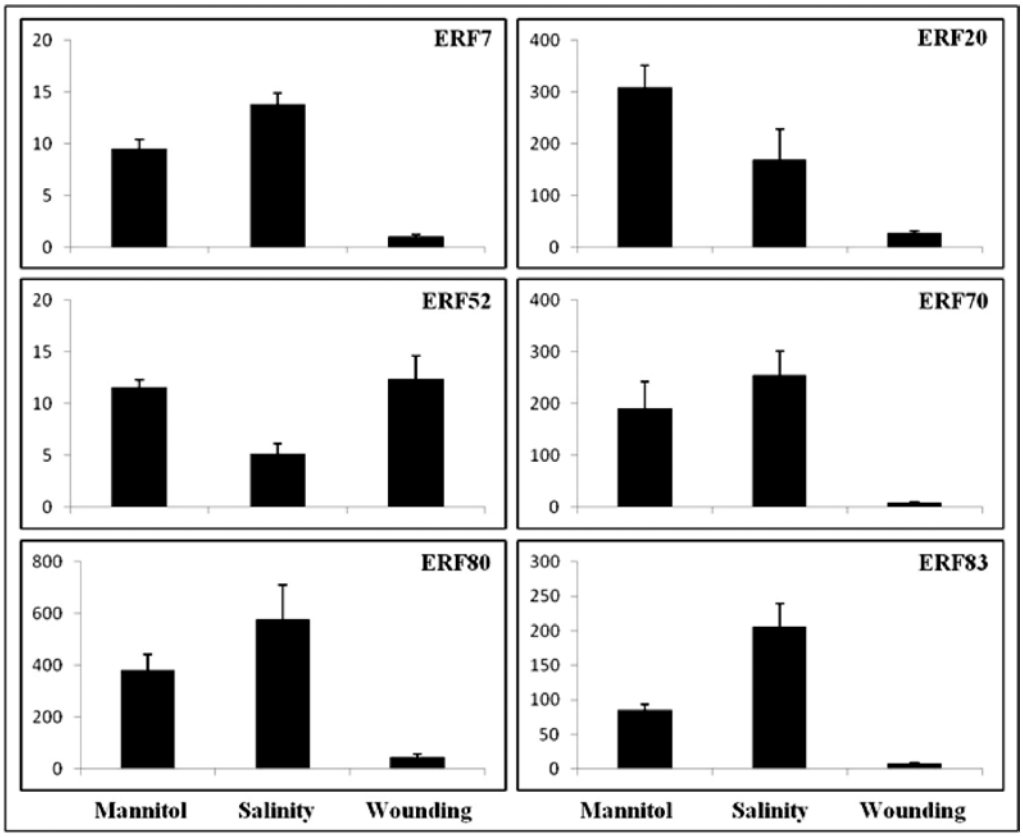


**Fig. 2.** Validation of *SmERF* family genes under mannitol, salinity and wounding treatments using qRT-PCR. Y-axis represents the fold change values at various stress conditions.

differential expression observed. Data presented in Fig. 3 showed differential expression of *SmERFs* during fruit development and ripening. *SmERF80* is the only gene which is highly up-regulated at 20 dpa and ripening stage (50 dpa). Expression of *SmERF52* and *SmERF83* was high at 5dpa but further down-regulated till ri pening. Expression of *SmERF7*, *SmERF70* and *SmERF80* was found to be high at ripening stage (50 dpa) where as the other three genes expression was equal to 0 dpa. *SmERF20* was found to be up-regulated at all the fruit developmental stages except 50 dpa.

In order to adapt to a large number of biotic and abiotic stresses, plants respond at physiological as well as biochemical levels. Many transcription factor families have been shown to exhibit stress-responsive gene expressions with significant overlap in response to various stress treatments, suggesting that signaling pathways involved in biotic and abiotic stresses are interconnected (Kunkel and Brooks, 8; Singh *et al.*, 14; Fujita *et al.*, 5). Present study is in accordance to the observations made by Sharma *et al.* (13) that *SlERF52* and *SlERF80* were up-regulated during various stresses. *SlERF70* was up-regulated 17 fold during salt and oxidation stress, whereas, *SlERF7* (DREB gene) was up-regulated in response to salt and drought stress. We have observed the induction of *SmERF* genes in response to all the three stresses suggesting a crosstalk between different stress signaling pathways. Over-expression of ERF family genes in *Arabidopsis*, tobacco and tomato has been shown to confer increased resistance to biotic as well as abiotic stresses (He *et al*., 7; Park *et al*., 10).

Ethylene plays a major role in the ripening of fleshy fruits. Understanding the key genes involved in ethylene biosynthesis and stress response is crucial to manipulate their expression for preventing losses due to over-ripening. ERFs regulate the expression of target genes in ethylene signal transduction pathway by binding to the GCC box in their promoter regions. These target genes in turn regulate the firmness, aroma, taste, colour and shelf Life of the fruits (Nath *et al.*, 9). In tomato, *SlERF7* and *SlERF80* exhibited high-level accumulation in immature green stage and gradually declined during fruit development (Sharma *et al.*, 13). In-contrast homologs of these genes

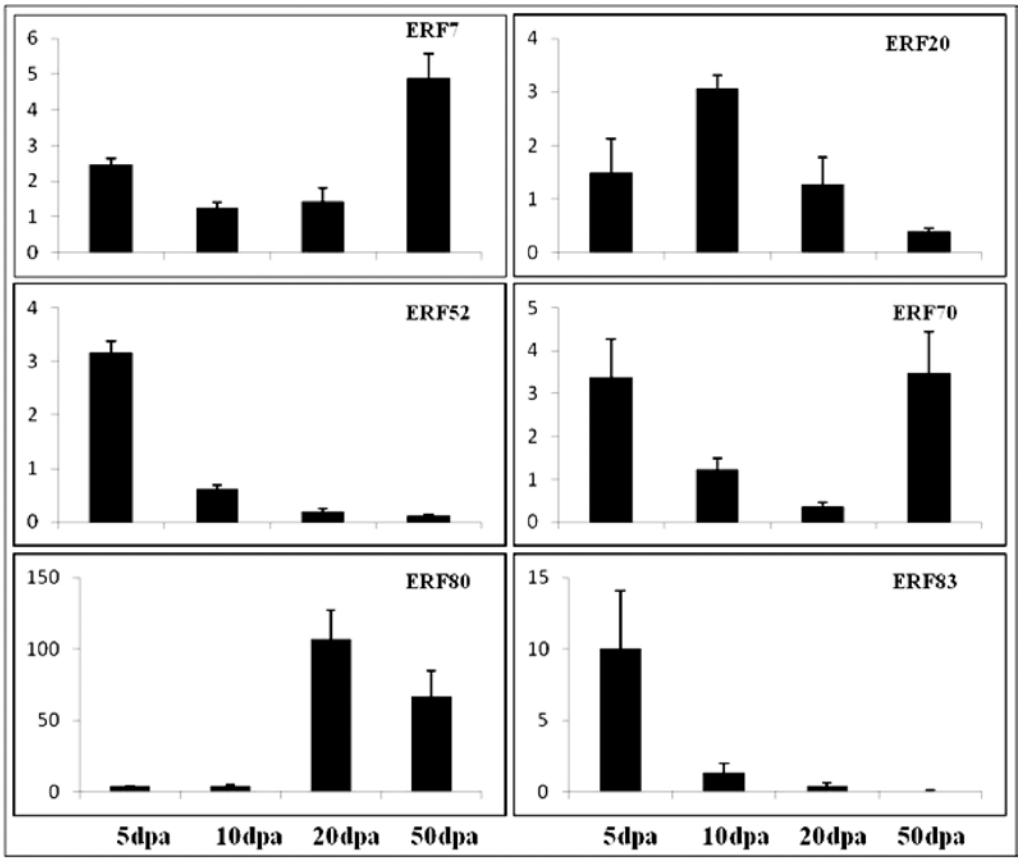


**Fig. 3.** Expression analysis of *SmERF* family genes under different fruit development stages (5, 10, 20 and 50 dpa) compared with 0 dpa. Y-axis represents the fold change.

*i.e. SmERF7* and *SlERF80* showed up-regulation at ripening stage in brinjal. Brinjal being a non-climacteric fruit, there is absence of sudden ethylene production at ripening stage. Probably up-regulation of these genes has some role in non-climacteric fruit ripening. The expression of two genes, *i.e*., *SmERF52* and *SmERF83* decreases during fruit development and ripening stage, which suggests that these ERFs solely require high ethylene level for their expression. Slight up-regulation of *SmERF7*, *SmERF20*, *SmERF52*, *SmERF70* and *SmERF83* at very early stage of development suggests its role in early stage of fruit development. In tomato, *SlERF83* was found to express only in red ripe stage of fruit development. *SlERF52* exhibited maximum expression specifically at immature green stage of fruit development with negligible expression in breaker and red ripe fruit stages. This shows the change in its role during evolution in tomato and brinjal. Expression of *SlERF70* was up-regulated from immature green to red ripe stages. Specific accumulation of their transcripts in different stages of fruit development indicates their involvement in stage-specific developmental activities (Sharma *et al.*, 13).

In conclusion, present study has shown differential expression and involvement of *SmERF7*, *SmERF20*, *SmERF52*, *SmERF70*, *SmERF80* and *SmERF83* under salt and drought stresses, and in response to mechanical wounding. *SmERF7*, *SmERF20*, *SmERF52*, *SmERF70* and *SmERF83* genes play important role at early stage of fruit development, whereas, *SmERF7*, *SmERF70* and *SmERF80* are probably involved in the non-climacteric ripening of brinjal fruit. This study opens the window of investigation of an important ERF transcription factor family of genes and its role in brinjal fruit development and stress responses.

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