J Biosci (2019) 44:137
DOI: 10.1007/s12038-019-9951-x

Molecular cloning and characterization of genes related to the ethylene signal transduction pathway in pomegranate (*Punica granatum* L.) under different temperature treatments

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MS received 23 March 2019; accepted 22 July 2019; published online 25 October 2019

Low temperature storage is a common method for storing pomegranates post-harvest; however, unsuitable low temperatures can cause fruit chilling injuries, the molecular mechanism of which is as yet unclear. Ethylene is a major factor affecting the post-harvest storage quality of pomegranates, and functions mainly through the ethylene signal transduction pathway. *ERF1*, *ERF2* and *ETR* are key genes in the ethylene signal transduction pathway. Here, we used RACE and homologous cloning techniques to obtain *PgERF1* (KU058889), *PgERF2* (KU058890) and *PgETR* (KU058891) from *Punica granatum* cv. Yushizi. Sequence alignment and functional domain analysis revealed that both PgERF1 and PgERF2 contained a DNA-binding-site at the 120th to 177th amino acids of the N-terminus, which is a typical AP2/ERF center structure domain. Analysis of changes in expression of *PgERF1*, *PgERF2* and *PgETR* following storage for different lengths of time (0, 14, 28, 42 and 56 days) at different temperatures (0°C, 5°C, 10°C and 15°C) revealed that the expression levels of *PgERF1* and *PgERF2* had a significant positive correlation. At the same time, the expression of both *PgERF1* and *PgERF2* increased continuously with time when seeds were stored at 0°C. However, there was no obvious linear relationship between time stored and the levels of expression of *PgETR*. Therefore, we inferred that at 0°C, the ethylene signal transduction pathway might play an important role in fruit chilling injuries during post-harvest storage.

Keywords. Cloning; ethylene; expression analysis; temperature treatments; post-harvest

1. Introduction

Pomegranate (*Punica granatum* L., family Lythraceae) plants are small deciduous trees or shrubs, and have high economic value due to the edible fruits, ornamental flowers and the exocarp, which is used in Chinese medicines (Chidambara Murthy *et al.* 2002; Lansky and Newman 2007; Singh *et al.* 2002;Yuan *et al.* 2017). Pomegranates are usually stored at low temperatures post-harvest. However, at low temperatures above 0°C, they are prone to chilling injuries including discoloration of aril and seed decay, leading to reduction of fruit quality, and at temperatures below 0°C, the fruits suffer frost injuries and irreversible damage (Zhang and Zhang 2009). Furthermore, pomegranate fruits suffer from chilling injuries, even if they are stored at temperatures below 5°C, and the symptoms of the chilling injuries will increase in severity with time spent at low temperatures (Fawole and Opara 2013). In consequence, post-harvest storage has become a bottleneck affecting the development of the pomegranate industry (Aviram and Dornfeld 2001; Negi et al. 2003). Pomegranate is non-climacteric fruit (Elyatem and Kader 1984) and ethylene is involved in regulating physiological changes of fruit ripening (White 2002). The objective of this study was therefore to investigate a potential molecular biological method about ethylene to improve the quality of post-harvest pomegranates.

Ethylene is a plant hormone, affecting senescence and stress resistance in plants, and is considered to be one of the factors that determine the characteristics of post-harvest pomegranates (Xu *et al.* 2014). Studies in mutants of the model plants *Arabidopsis thaliana* and *Nicotiana tabacum* suggest that *ERF1*, *ERF2* and *ETR* are important genes in the ethylene signal transduction pathway.

ERFs (Ethylene-response factors) comprise one of the largest transcription factor gene families, and 122 ERF genes, grouped into 12 subfamilies, have been found in *Arabidopsis* (Nakano *et al.* 2006). *ERFs* have been isolated from several other species of plants, including *Prunus mume* (Du *et al.* 2012), *Prunus persica* (Zhang *et al.* 2012), *Malus pumila* (Girardi *et al.* 2013), and *Citrus reticulata* (Xie *et al.* 2014). *ERFs* are positive regulatory elements in the ethylene pathway and are located in the nucleus. Two *ERF* binding elements have been found: the GCC-box (core sequence AGCCGCC), and DREB (core sequence CCGAC) (Riechmann *et al.* 2000; Sun *et al.* 2008). The *ERF* family, which

has a conserved AP2 region, comprises downstream components of the ethylene signal transduction pathway. Expression controlled by the AP2 region is directly regulated by EIN3 and the genes in the ERF family can bind to ethylene inducible gene promoter GCC-box, promoting downstream ethylene response gene (Bie *et al.* 2013).

ETRs (Ethylene receptors) are the upstream components of the ethylene signal transduction pathway and function by binding ethylene, in turn affecting the physiological functions of plants (O'Malley *et al.* 2005). *ETRs* are proteins with high homology with bacterial two-component histone proteins. Their basic structure includes a C-terminal histidine kinase domain, an N-terminal ethylene binding domain and a reaction regulation domain (Chang and Stadler 2001). In *Arabidopsis thaliana*, there are five ETR proteins, ETR1, ETR2, ERS1, ERS2 and EIN4. These five receptors play negative regulatory roles in ethylene signal transduction (Alonso *et al.* 2003). Since Wilkinson *et al.* (1995) isolated the *LeETR3* gene from tomato (*Lycopersicon esculentum*), *ETR* genes have been isolated from several different fruits, including apple (*Malus pumila*) (Tatsuki *et al.* 2007), strawberry (*Fragaria ananassa*) (Trainotti *et al.* 2005), and citrus (*Citrus reticulata*) (Katz et al. 2004).

In this study, we cloned and characterized three genes related to the ethylene signal transduction pathway (*PgERF1*, *PgERF2* and *PgETR*) from pomegranate. Following analysis of different levels of expression of *PgERF1*, *PgERF2* and *PgETR* under different temperature treatments and subjected to storage for different lengths of time, we observed that the expression levels of *PgERF1* and *PgERF2* in pomegranate fruits increased under low storage temperatures, and is correlated with increasing chilling injuries. These genes therefore may play an important role in fruit chilling injuries sustained during post-harvest storage. However, there was no obvious linear relationship between expression levels of *PgETR* under different temperature treatments, suggesting that the expression level of *PgETR* has no direct effect on the resistance of pomegranates to low temperatures. This work lays the foundation for further research into analysis of the functions of *PgERF1*, *PgERF2* and *PgETR,* and also provides gene resources for the breeding of new storage-resistant cultivars of pomegranate.

2. Materials and methods

2.1 *Plant materials and pretreatments*

The pomegranates used in this study were of the cultivar, 'Yushizi', and were collected from a fruiting pomegranate tree growing at the pomegranate germplasm bank of Anhui Agricultural University. Fruits were collected, and the peel and arils were manually removed from the grain, leaving only the seed. The seed were then plunged into liquid nitrogen, taken back to the laboratory and stored at −80°C. Finally, the materials were placed in 4°C for 72 h, and then divided into four groups, which were kept in petri dishes for 56 days at 0°C, 5°C, 10°C and 15°C, respectively.

2.2 *Extraction of RNA and DNA*

In order to investigate genes related to the ethylene signal transduction pathway, total RNA was extracted from the seed samples previously stored at different temperatures. Seed samples were ground using a grinding rod, and total RNA was extracted from the samples with the StarSpin Plant RNA Mini Kit (GenStar, China), according to the manufacturer's instructions. RNA samples were then treated with 16 μl DNase I (GenStar, China) to eliminate contamination with genomic DNA. A M-MLV RTase cDNA Synthesis Kit (TaKaRa, Japan) was used to obtain first-strand cDNA from the purified total RNA. At the same time, genomic DNA was extracted using the Plant Genomic DNA Kit (Tiangen, China).

2.3 *Isolation of the full-length of PgERF1, PgERF2 and PgETR genes*

cDNA was synthesized using M-MLV reverse transcriptase (TaKaRa, Japan), using the total RNA as the template. Degenerate primers for the *ERF1*, *ERF2* and *ETR* genes were designed based on sequences of several species logged in NCBI (*https://www.ncbi.nlm.nih.gov*), to obtain intermediate fragments. Subsequently, using the intermediate fragment sequences, 5′ end amplified specific primers and 3′ end specific primers were designed. The SMARTer^{TM} RACE cDNA Amplification Kit (TaKaRa, Japan) was used together with the primers we designed, we obtained the 5′ end and 3′ end sequence of genes. Then the three parts were spliced, and new primers ERF1-F/ERF1-R, ERF2-F/ERF2-R and ETR-F/ETR-R were designed to amplify the full-length sequence (table 1). Finally, *PgERF1, PgERF2* and *PgETR* were amplified using KOD polymerase (TOYOBO, China), followed by terminal adding A reaction and attached to the Blunt Simple vector (Tiangen, China), and attached to the Blunt Simple vector (Tiangen, China), then sequenced by Sangon Biotech.

2.4 *Bioinformatics analysis*

Sequence analysis and comparison with known sequences were performed using the NCBI BLAST server (*http://www.ncbi.nlm.gov/BLAST*). The physical and chemical properties of the proteins were calculated using the PeptideMass program (*http://us.expasy.org/tools/peptide-mass.html*) (He *et al.* 2013). ProtCompVersion 9.0 (*http://linuxl.Softberry.com/berry.phtml*) was used to predict the subcellular localization of the proteins. In order to compare the homology among various ERF1, ERF2 and ETR proteins, an alignment was

Table 1. Primers for gene cloning and qRT-PCR analysis

Primer	(5′ → 3′) Sequence	Usage
UPM Long	CTAATACGACTCACTATAGGGCA AGCAGTGGTATCAACGCAGAGT	Universal primers
NUP	AAGCAGTGGTATCAACGCAGAGT	
ERFl-3′GSP1	GTATCCTCCATCCTTTCAGC	Amplification for 3′-RACE of *PgERFl*
ERFl-3′GSP2	AAGCAGTGGTATCAACGCAGAG	
ERFl-5′GSP1	TGAGAATCAAATGCCGAGAG	Amplification for 5′-RACE of *PgERFl*
ERFl-5′GSP2	ACGCAAACTGATTCACTGGG	
ERFl-F	TTCTTCGCCGCCCCCAAGTCTC	Amplification for full length of *PgERFl*
ERFl-R	TACATAAAACGAGCGAGCACAA	
ERFl-RT-F	GACTCAAGATGGCGGGAATG	Gene-specific primers for *PgERFl* Real-time PCR
ERFl-RT-R	AAAACGAGCGAGCACAAAGA	
ERF2-3′GSP1	CTCTGCGGGTTTCTTGGA	Amplification for 3′-RACE of *PgERF2*
ERF2-3′GSP2	AAGCAGTGGTATCAACGCAGAG	
ERF2-5′GSP1	TCTGAGAATCAAATGCCGAG	Amplification for 5′-RACE of *PgERF2*
ERF2-5′GSP2	CGCTTTGACGAGGAGTTAGG	
ERF2-F	AACGGGGACACGACTCAAGA	Amplification for full length of *PgERF2*
ERF2-R	TAAAACGAGCGAGCACAAAG	
ERF2-RT-F	AATGGGCTGCTGAAATACG	Gene-specific primers for *PgERF2 R*eal-time PCR
ERF2-RT-R	ATGGAGGATACATCGGGAGT	
ETR-3′GSP1	GCATACTTCTCCATCCCCTT	Amplification for 3′-RACE of *PgETR*
ETR-3′GSP2	AAGGCTGTTGCTGTGGTGAT	
ETR-5′GSP1	GAAACAGCAGCACAAGAC	Amplification for 5′-RACE of *PgETR*
ETR-5′GSP2	ACCACAGCAACAGCCTTC	
ETR-F	CCTACAGATGGGTTCTAATGC	Amplification for full length of *PgETR*
ETR-R	TTTGGTGGTGAAGAGTATGTC	
ETR-RT-F	ATCGGCATTCTTCCCCTACA	Gene-specific primers for *PgETR* Real-time PCR
ETR-RT-R	CACCACAGCAACAGCCTTCG	
PgACT-PF	AGTCCTCTTCCAGCCATCTC	Amplification for *PgACTIN*
PgACT-PR	CACTGAGCACAATGTTTCCA	

Table 2. Physical and chemical characteristics of PgERFs and PgETR

Characteristic	PgERF1	PgERF2	PgETR
Molecular formula	C1927H2933N537O622S9	C1929H2936N536O625S9	C1009H1589N255O274S16
Number of amino acids	394	394	193
Molecular weight (kDa)	43.86	43.92	22.18
Theoretical PI	4.97	4.93	5.85
Grand average of hydropathicity	−0.890	−0.901	0.364
Number of amino acids	394	394	193
Sub-cellular localization (score)	Nucleus (9.8)	Nucleus (9.7)	Plasma membrane (6.0)

performed using CLC Sequence Viewer 6 software. Full-length amino acid sequences were aligned using ClustalW and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 5.01 (Tamura *et al.* 2011). Phylogenetic analyses were conducted using a neighbor-joining method in MEGA. Bootstrap tests were conducted using 1,000 replicates, and the branch lengths are proportional to the phylogenetic distances (Saitou and Nei 1987).

2.5 *Real-time PCR quantification analysis*

Two ug of RNA from each of the different samples digested by DNase I was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. Real-time quantitative PCR was performed using an ABI 7500 Fast Real-Time PCR system and SYBR®Premix Ex Taq™ II (TaKaRa, Japan), and a Real-Time PCR system following the manufacturer's instructions. The PCR reaction mixture (25 μL total volume) contained 12.5 μL 2 × SYBR® Premix Ex Taq™ (TaKaRa, Japan), 0.5 μL of each primer (10 μM), 2 μL of tenfold diluted cDNA and 9.5 μL ddH_2O. Amplification was carried out using an initial denaturation step at 95°C for 30 s, followed by 40 cycles of amplification (denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 1 min). The results were analyzed using SPSS Statistics software, and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). *PgACTIN* was used as the reference gene.

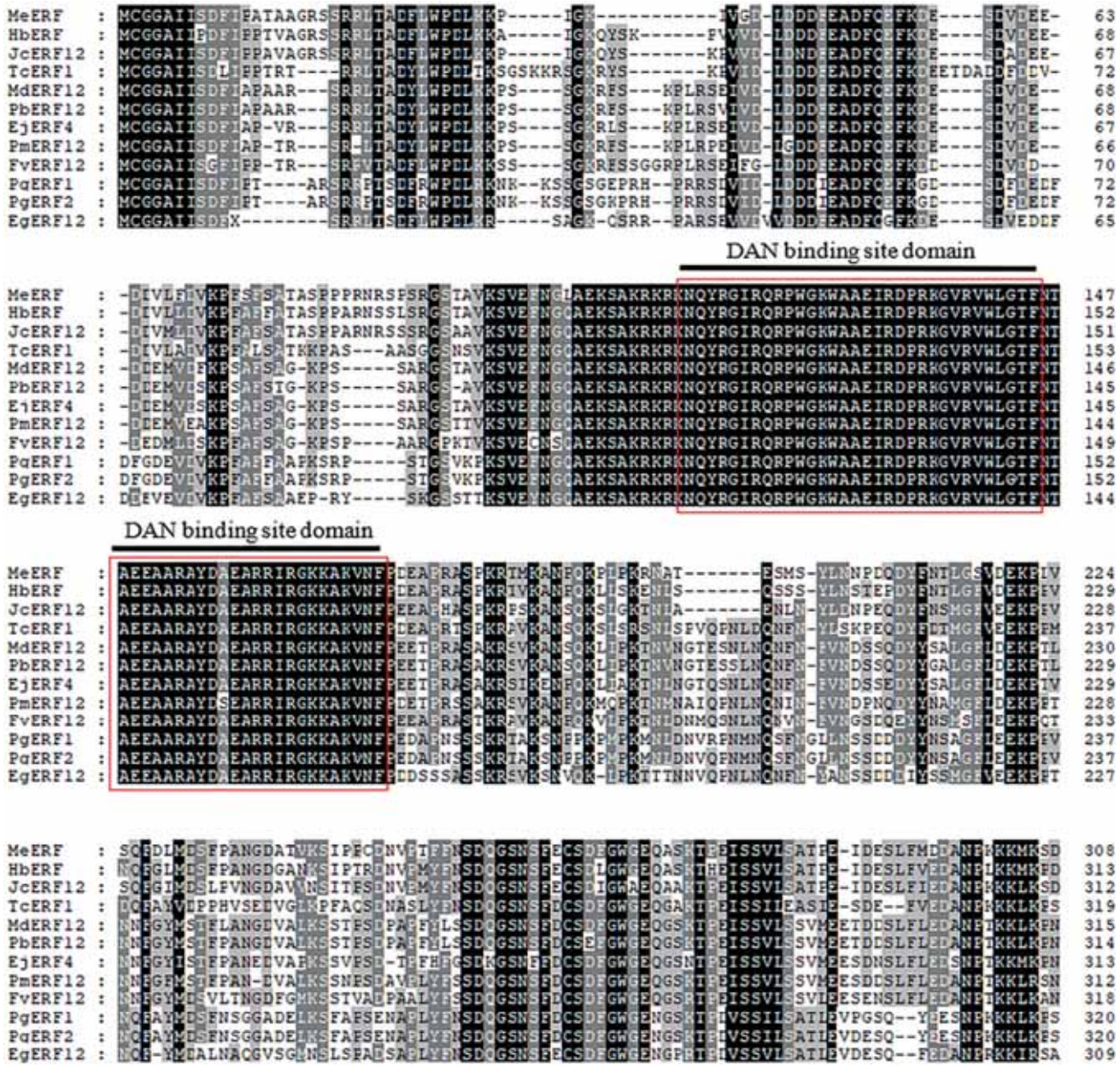

Figure 1. Alignment of PgERF amino acid sequences with those of ERFs from other plant species. The amino acid sequence in the maps represent the following species (from top to bottom): *Manihot esculenta* (AAX84670.1), *Hevea brasiliensis* (AFY09702.1), *Jatropha curca* (NP_001295665.1), *Theobroma cacao* (XP_007024746.1), *Malus domestica* (NP_001280911.1), *Pyrus* × *bretschneider* (XP_009357932.1), *Eriobotrya japonica* (AFG26329.1), *Prunus mume* (XP_008228117.1), *Fragaria vesca* subsp (XP_011467483.1). vesca, *Punica granatum* (PgERF1 KU058889), *Punica granatum* (PgERF2 KU058890), *Eucalyptus grandis* (XP_010029503.1).

2.6 *Statistical analysis*

Data from at least three independent experiments were analyzed using one-way ANOVA in Sigmaplot v11.0 (Jandel Scientific Software, San Rafael, CA, USA), and the differences were compared using a Duncan test with a significance level of P\0.05

3. Results and discussion

3.1 *Cloning and sequence analysis of PgERF1, PgERF2 and PgETR*

Full-length coding regions (CDSs) of the *PgERF1*, *PgERF2* and *PgETR* genes were amplified from the cDNA samples

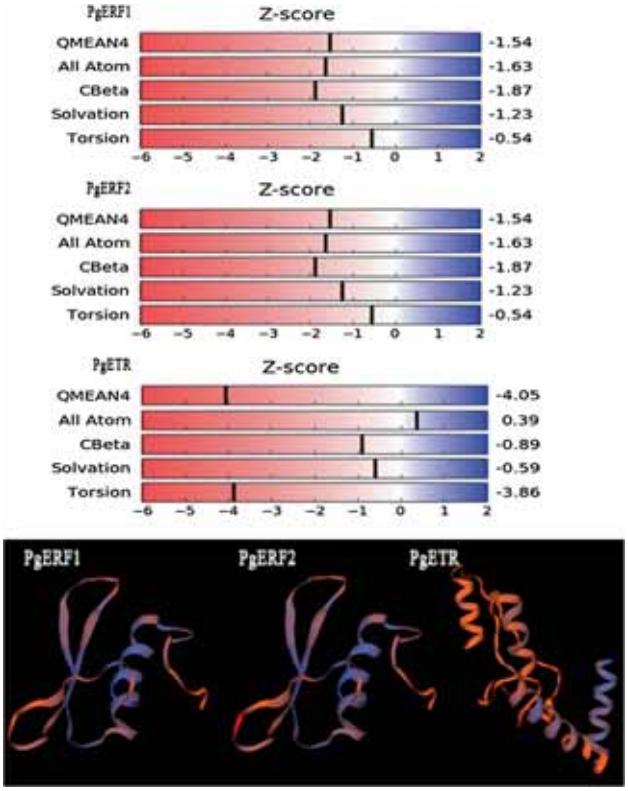

Figure 2. Homology modeling of the PgERFs and PgETR. (Structural homology models were generated in SWISS-MODEL.)

reverse transcribed from total RNA. The genes were named based on their similarity to those from other species and the order of their discovery. Gene sequence analysis indicated that the CDSs of PgERF1, PgERF2 and PgETR encoded 394, 394 and 420 amino acid residues with predicted molecular masses of 43.86, 43.92 and 22.18 kDa, respectively. The predicted theoretical PI were 4.97, 4.93 and 5.83, and the molecular formulae of the proteins were $C_{1929}H_{2936}N_{536}O_{625}S_9$, $C_{1929}H_{2936}N_{536}O_{625}S_9$, and $C_{1009}H_{1589}N_{255}O_{274}S_{16}$, respectively (table 2). Prediction of the hydrophobicity of the deduced amino acid sequences indicated that the GRAVY values of ERF1 and ERF2 were above zero, while that of ETR was below zero. ERF1 and ERF2 were predicted to be located in the nucleus, with instability parameters of 46.50 and 45.42, respectively. However, ETR was predicted to be located in the membrane, and had an instability parameter of 44.76.

Sequence alignment and functional domain analysis suggested that both PgERF1 and PgERF2 contained a single DNA-binding-site domain, located at the N-terminal 120-177 amino acid position, as a typical AP2/ERF center domain (figure 1). PgETR displayed high similarities with other ETR protein sequences. For example, the similarity between PgETR and McETR, FsETR1, PtETR reach to 93.51%, 92.54% and 91.11%, respectively. Predictions of the tertiary protein structures of PgERF1, PgERF2 and PgETR were generated in SWISS-MODE (figure 2).

3.2 *Phylogenetic analysis and homology protein analysis*

In order to determine the phylogenetic relationships between these three genes, MEGA software was used for multiple sequence alignment and subsequent phylogenetic analysis. The results suggest that PgERF1 and PgERF2 are indeed homologues of the ERF family, and have a high degree of sequence similarity with the ERFs in other species. PgERF1 and PgERF2 fell in a single clade together with EgERF12 (Accession: XP_010029503.1), and these three shared the highest homology of all the sequences assessed. However, PgERF1 and PgERF2 were grouped far from PbERF12 (Accession: XP_009357932.1) in the resulting phylogenetic tree (figure 3).

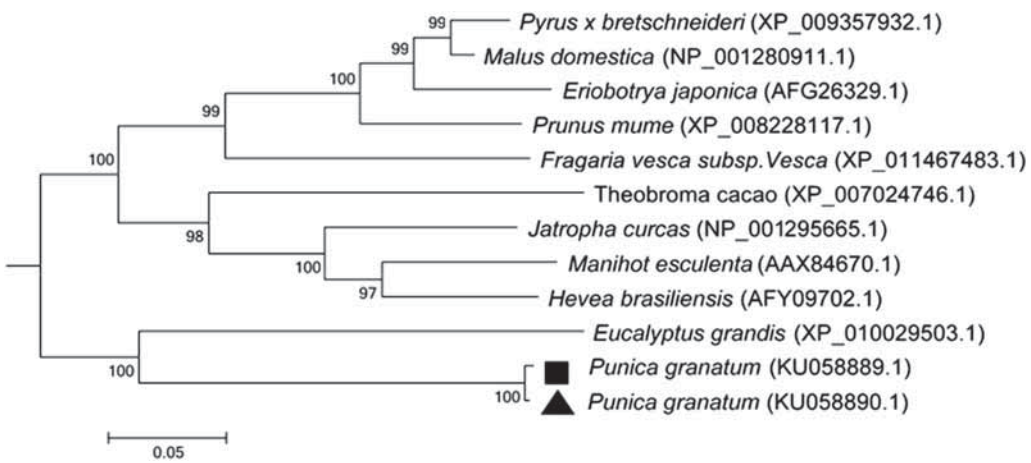

Figure 3. Phylogenetic tree comparing the amino acid sequences of PgERF1 and PgERF2 to those of ERFs from other species. Bootstrap values greater than 30% are shown at nodes based on 1000 replications; The scale bar represents genetic distance; The accession number is given in brackets.

An evolutionary tree based on amino acid sequence similarity between PgETR and similar proteins from other plants was also constructed (figure 4). In the resulting tree, PgETR fell in a clade with CsETR (Accession: AAC99435.1), with which it had high homology, but that it was not closely related to PsETR (Accession: CAA06723.1). However, PgETR shared high sequence similarity with ETR proteins from other species.

3.3 *Expression analysis of PgERF1 under different temperature scenarios*

Samples stored post-harvest for different lengths of time (0 d, 14 d, 28 d, 42 d and 56 d) were subjected to different temperature treatments (0°C, 5°C, 10°C and 15°C) in order to investigate the relative expression of the genes *PgERF1*, *PgERF2* and *PgETR*.

The results showed that in samples stored at 0°C, the expression level of *PgERF1* increased continuously from 0 to 56 days, reaching the highest level of expression, which was 2.83 times that of day 0, on day 56. Samples stored at 5°C showed increasing *PgERF1* expression levels over the first 28 days, reaching the highest level of expression (1.6 times that on day 0) on day 28 dayd. Expression levels of *PgERF1* then decreased with further storage time. Samples stored at 10°C and 15°C showed significant increases in expression of *PgERF1* from 0 d to 14 days, reaching the highest levels of expression (1.65 and 1.81 times that of day 0, respectively) on day 14. Expression levels then decreased from day 14 to day 42, and rose again between day 42 and day 56. In general, over the 56 days of storage, the expression levels of *PgERF1* increased with increasing storage time when the samples were kept at 0°C. When samples were stored at 5°C, 10°C and 15°C, *PgERF1* expression increased at first, reaching the highest levels of expression on days 28, 14 and 14 respectively, and then decreased to varying degrees (figure 5).

3.4 *Expression analysis of PgERF2 under different temperature scenarios*

The results showed that in samples kept in storage at 0°C, the levels of expression of *PgERF2* also increased continuously from day 0 to day 56, reaching the highest level of expression (3.12 times that of day 0) on day 56 d. When samples were stored at 5°C, *PgERF2* expression increased at first, reaching the highest level (1.26 times that on day 0) on day 28, following which it decreased (0.99 times that on day 0). When samples were stored at 10°C and 15°C, the levels of expression of *PgERF2* in all samples increased significantly from day 0 to day 14, reaching a maximum level of expression (1.96 and 2.32 times that of day 0, respectively) on day 14. Expression levels then decreased from day 14 to day 42, and rose again between day 42 and day 56. In general, during the 56 days of storage, the expression levels of *PgERF2* increased with increasing storage time when kept at 0°C. When samples were stored at 5°C, *PgERF2* expression increased initially and then decreased. Samples stored at 10°C and 15°C, showed initial increases in *PgERF2* expression, which reached maximum expression on day 14, and then decreased to varying degrees (figure 6).

Correlations between *PgERF2* expression and *PgERF1* expression in pomegranate grains stored at different temperatures were analyzed using SPSS software. The results suggest that there was a significant positive correlation between *PgERF2* and *PgERF1* expression ($r = 0.954$, $P<0.01$).

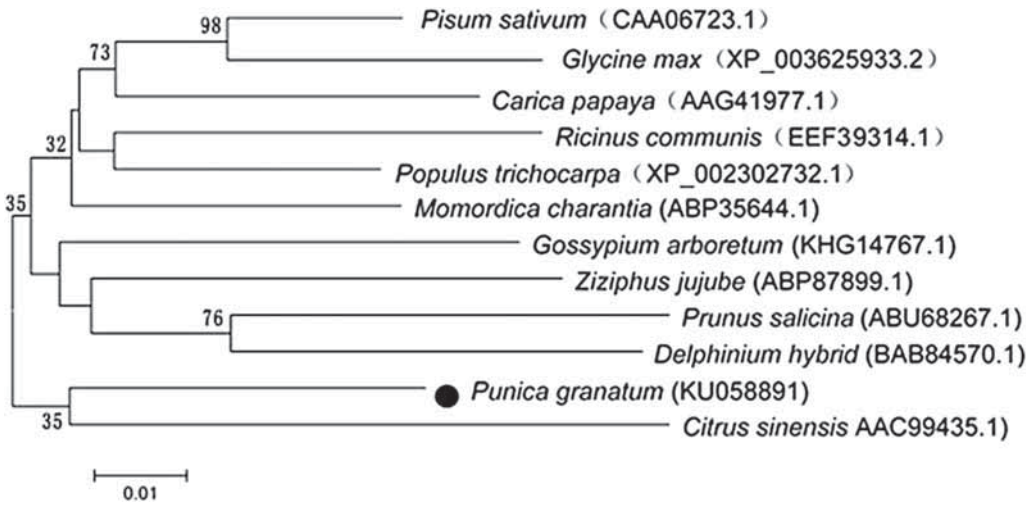

Figure 4. Phylogenetic tree comparing the amino acid sequences of PgETR to ETR from other species. Bootstrap values greater than 30% are shown at nodes based on 1000 replications; The scale bar represents genetic distance. The accession number was given in brackets.

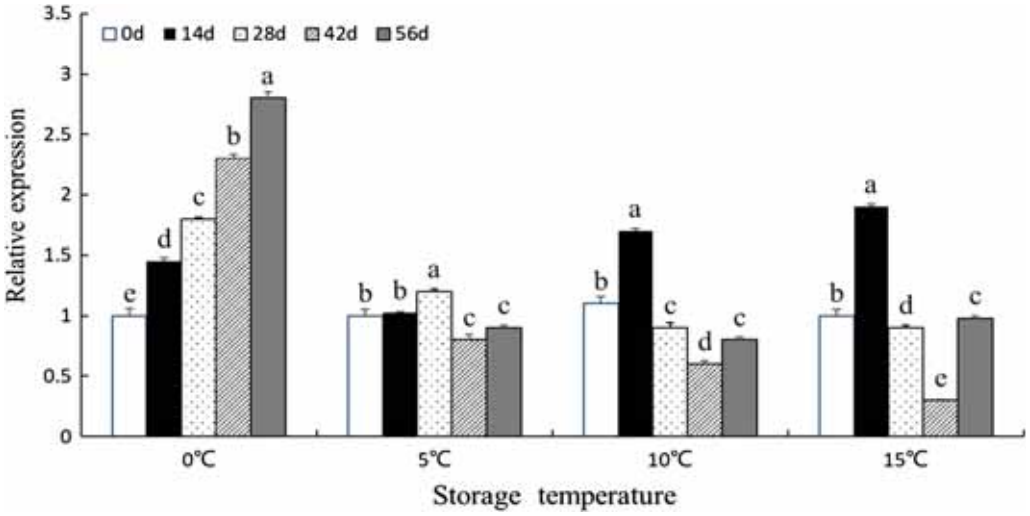

Figure 5. Relative expression of PgERF1 in pomegranate seeds stored at different temperatures. Within each temperature treatment group, bars are marked with lower case letters to indicate statistically significant differences. Where there is no significant difference between bars, these are marked with same letter.

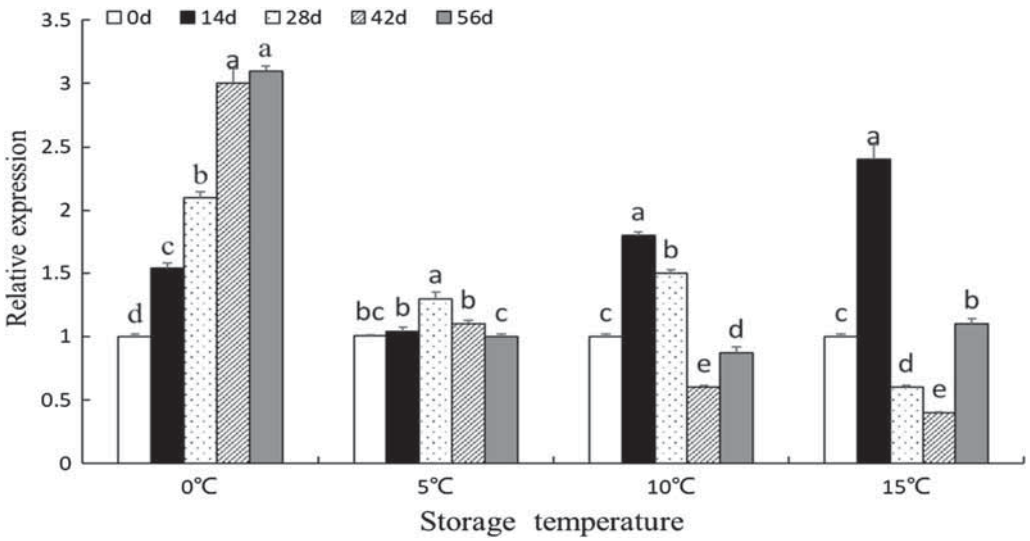

Figure 6. Relative expression of PgERF2 in pomegranate seeds stored at different temperatures. Within each temperature treatment group, bars are marked with lower case letters to indicate statistically significant differences. Where there is no significant difference between bars, these are marked with same letter.

3.5 *Expression analysis of PgETR in seeds stored at different temperatures*

The results showed that in samples stored at 0°C, the expression levels of *PgETR* increased rapidly from day 0 to day 14, reaching a maximum level of expression (1.94 times that of day 0) on day 14. Following this maximum, *PgETR* expression decreased between day 14 and day 28, and between day 28 and day 56 there was a small increased. In samples stored at 5°C, *PgETR* expression levels also increased significantly from day 0 to day 14, reaching a maximum (2.20 times that of day 0) on day 14, and then subsequently decreased from 14 to 28 days. *PgETR* Expression levels then increased between day 28 and day 42, and decreased again between day 42 and day 56. When samples were stored at 10°C for 56 days, *PgETR* expression first increased to a maximum level (2.25 times that of day 0) on day 14, then decreased between days 14 and 42, and finally increased again significantly. In contrast, in samples stored at 15°C for 56 days, the expression levels of *PgETR* at first decreased slightly between days 0 and 14, then increased to a maximum level (3.10 times that of day 0) on day 42, then finally decreased once more (figure 7).

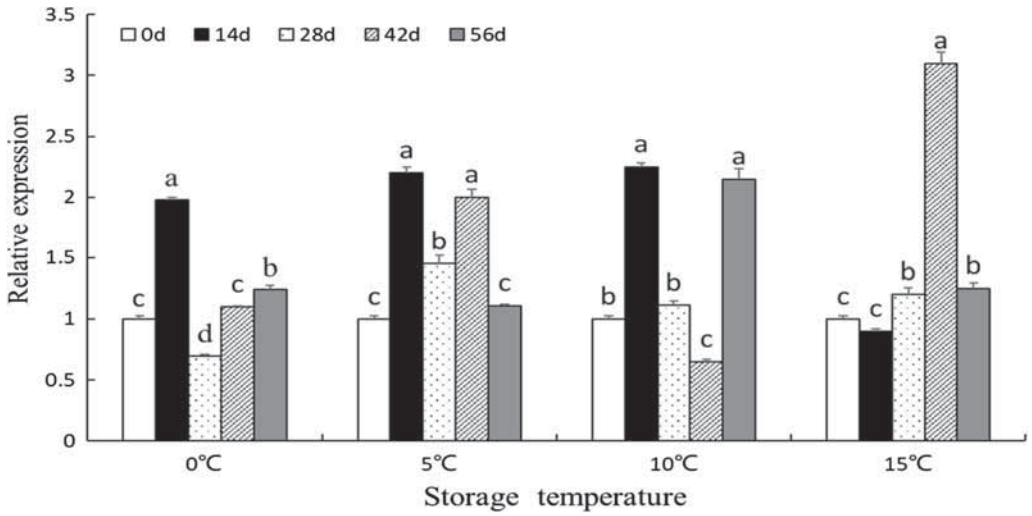

Figure 7. Relative expression of PgETR in pomegranate seeds stored at different temperatures. Within each temperature treatment group, bars are marked with lower case letters to indicate statistically significant differences. Where there is no significant difference between bars, these are marked with same letter.

4. Conclusions

PgERF1, *PgERF2* and *PgETR* genes related to ethylene signal transduction pathway were isolated and characterised from the pomegranate cultivar 'Yushizi' in this experiment. Following amino acid sequence alignment and phylogenetic tree analysis, it was found that both PgERFs and PgETR contained conserved regions. The amino acid sequences of the conserved regions in these ERFs are highly consistent with those from different species. However, differences in the non-conserved regions are very large, which may lead to the observed differences in physicochemical properties of the ERF amino acids from different species (Jing *et al.* 2008). The AP2/ERF transcription factor family is a class of transcription factors found across many different plants. All members of this family contain highly conserved DNA domains comprising 60–70 amino acid residues, namely AP2/ERF domains (Jing *et al.* 2008). Both PgERF1 and PgERF2 proteins contain a DNA-binding-site domain at the N-terminal 120-177 amino acids, which is a typical AP2/ERF central domain. Therefore, it can be inferred that *PgERF1* and *PgERF2* are AP2/ERF transcription factor family genes. According to reports by Chung *et al.* (2010) and Lee *et al.* (2012), following fruit ripening in transgenic tomato (*Lycopersicon esculentum*), ethylene synthesis increased together with *SlAP2a*, which is an AP2/ERF member gene.

Quantitative fluorescence analysis of genes related to ethylene signal transduction showed that *PgERF1, PgERF2* and *PgETR* all were expressed in pomegranate grains following storage for different lengths of time at different temperatures. The patterns of change observed in the expression levels of *PgERF1* and *PgERF2* have similarities. Relevant studies have suggested that ethylene signal transduction genes have the ability to change fruit stress resistance: four ERF genes of *Chaenomeles sinensis* all respond to low temperature (7°C) and high temperature (35°C) (Lee *et al.* 2012). The expression of *AdERF3*, *AdERF4*, *AdERF11*, *AdERF12* and *AdERFl4* in *Actinidia chinensis* increased continuously at both low temperature (0°C) and high temperature (35°C) (Lee *et al.* 2012). Our results suggest that the expression of *PgERF1* and *PgERF2* increased continuously for 56 days when samples were stored at 0°C. Therefore, we conclude that the increases in expression of *PgERF1* and *PgERF2* under conditions of low-temperature stress in pomegranate fruits play an important role in the chilling injuries shown by pomegranate fruits during post-harvest storage, which is consistent with the results of Zhang and Huang (2010) and Yin *et al.* (2012). Studies on non-climacteric loquat fruits showed that *EjETR1* can response to chilling stress (Wang *et al.* 2010). However, we did not find any obvious linear relationship between the levels of expression of *PgETR* and different temperature treatments in this experiment. Therefore, we deduce that *PgETR* may not be sensitive to low temperature and has little effect on the post-harvest quality of pomegranate. However, we believe that *PgERF1* and *PgERF2* may play important roles in pomegranate post-harvest storage.

The ethylene signal transduction conductivity model was established through genetic and biochemical studies on mutants of *Arabidopsis thaliana* and *Nicotiana tabacum*, and has five major components: ETRs, CTR1, EIN2, EIN3/EILs and ERFs (Yin 2009). Every component is made up of proteins from multiple sets of genes and with different functions, and the effects of low temperature stress on other genes in the ethylene signal transduction pathway need further study. For example, ethylene receptors, including *ETR1*, *ETR2*, *ERS1*, *ERS2*, *EIN4*, act as negative regulators of ethylene signals. Ethylene receptors can relieve the inhibition of downstream genes by restraining the downstream

negative regulator CTR1 to activate the positive regulator EIN2 in the cytoplasm. Transfer signals are then sent to the transcription factor EIN3/EILs in the nucleus, promoting the expression of transcription factor ERFs, and finally regulating the expression of related genes. The function of *PgERF1*, *PgERF2* and *PgETR* is therefore worth in-depth analysis to enable elucidation of the molecular mechanism of how ethylene effects low temperature stress resistance in pomegranates. This research also lay a theoretical foundation for the cultivation of new storage-resistant cultivars of pomegranate.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant number 30900971).

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Corresponding editor: Manchikatla Venkat Rajam