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Identification, characterization and expression analysis of ERF transcription factor *VviERF073* and standardization of stable reference genes under salt stress in grape

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Summary

Salinity is one of the several abiotic factors affecting grape productivity and quality. Transcription factors belonging to AP2/ERF family play an important role in abiotic stress response. Based on the *in silico* analysis of salt stress libraries of grape ESTs, a transcription factor *VviERF073*, having a single AP2/ERF conserved domain was identified. The sequence of *VviERF073* was analyzed in 'Thompson Seedless'. *VviERF073* belonged to the B2 subfamily of ERF transcription factors. Under salt stress, the relative expression of *VviERF073* increased 2.7 fold in the leaves of grafted grapevine within six hours of salt stress. The expression increased to 8.7 fold at 15 days of salt stress, thus indicating its role in early as well as late response to salt stress. However, expression of *VviERF073* did not change significantly in grapevines raised on own roots. The expression varied in the roots of 'Thompson Seedless' but not in the roots of '110R'. Under salt stress conditions, *GAPDH* and *EF1α* for grafted vines and *Actin* and *Tubulin* for own root vines, were identified as the stable reference genes for qPCR data normalization.

K e y w o r d s : grape; AP2/ERF; *VviERF073;* salt stress.

Introduction

Plants face several abiotic stresses like soil salinity, drought, extreme temperatures, and radiation etc. during their life cycle. Soil salinity is considered to be a major constraint to improve food production. Salinity not only affects yield but also reduces arable area. According to an estimate, about 20 % of agricultural land in the world is saline (Flowers and Yeo 1995). Due to their sessile nature, plants evolve several strategies to survive under biotic and abiotic stresses (Munns and Tester 2008). Salt stress is a genetically complex trait, often modulated by multiple biosynthetic and signaling pathways.

Grape (*Vitis vinifera* L.) is one of the most economically important fruit crops in the world and is mainly used for the production of wine and distilled liquor products. It is also used for fresh consumption, raisin, juice, grape seed oil and processed for pharmaceutical uses. Grape is mainly grown in semi-arid regions and thus suffers not only water deficit but also is prone to salinization (Cramer 2010). Various workers have ranked grape as sensitive (Prior *et al.* 1992) to moderately sensitive (Shani *et al.* 1993) to salinity stress. Early effects of salt stress in grapevine result in reduced photosynthesis rate and stomatal conductance leading to subsequent loss of vigor and yield (Walker *et al.* 2010). To confer improved salt tolerance to scion variety, several *Vitis* species and their hybrids are used as rootstocks for commercial grape production. These rootstocks have sodium and chloride ion exclusion properties. Rootstock '110 Richter' ('110R') has been shown to have Na and Cl exclusion properties under Indian grape growing conditions (Upadhyay *et al.* 2013) and considerably improves performance of table grape varieties under saline soil conditions.

Plant response to abiotic stresses *i.e.* drought, salinity, extreme temperature and radiation is modulated by regulation of gene expression by transcription factors (Singh *et al.* 2002). Putative role of several transcriptional factors under stress conditions has been studied in different crops. The APETALA2/ethylene-responsive element binding factor (AP2/ERF) family containing well conserved domain region consisting of 50-60 amino acids, is a large group of plant-specific transcription factors involved in abiotic stress responses by regulating gene expression (Mizoi *et al.* 2012). AP2/ERF TFs have been identified in various plant species like Arabidopsis, rice, tomato, soybean, wheat and grape (Nakano *et al.* 2006, Xu *et al.* 2007, Zhuang *et al.* 2009). This family comprises of five sub families *i.e.* AP2, RAV, ERF, DREB and other proteins (Sakuma *et al.* 2002). Among these, transcription factors belonging to the ERF subfamily containing a single conserved domain have been shown to be involved in salinity stress response in many plant species (Ayarpadikannan *et al.* 2014, Dong *et al.* 2012). These transcription factors recognize and bind to core GCCGCC sequence present in the promoters of target genes (Ohme-Takagi and Shinshi 1995).

In grape, genome wide analysis identified 132 putative genes belonging to AP2/ERF family. These genes were classified into four subfamilies *i.e.* AP2, DREB, ERF, RAV

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and a soloist (Zhuang *et al.* 2009). In another study, Licausi *et al.* (2010) reported 149 sequences belonging to AP2/ERF superfamily and analyzed their expression in vegetative and reproductive tissues. Zhu *et al.* (2013) reported role of three ERF factors from *Vitis pseudoreticulata* in different biotic and abiotic stress-responsive pathways. However, the role of ERF TFs in salinity stress response in grape is not studied so far. In this paper, we describe the identification of a salt stress responsive ERF TF based on *in silico* analysis of salt stress ESTs of grape. This ERF TF was identical to *VvERF057* reported by Licausi *et al.* (2010) and has been annotated as *ERF073* in NCBI *Vitis vinifera* Annotation Release 101 (updated December 2014). We named this gene as *VviERF073* following the gene nomenclature system suggested by Grimplet *et al.* (2014). We report the sequencing and characterization of *VviERF073*, its expression analysis in leaf and roots of 'Thompson Seedless' variety of table grape in response to salt stress. Expression of this gene was also analyzed in different parts of field grown vines of 'Thompson Seedless'. We also compared expression of this gene in own-rooted and grafted grapevines to study the effect of rootstock on its expression under salt stress. Though several reports have described the stability of reference genes in different vegetative and reproductive stages of grapevines as well as biotic and abiotic stresses, these reports pertain to grapevines raised on own roots. We have not come across any report on the effect of salt stress and rootstock on the expression of reference genes. Therefore, the most stable reference genes suitable for qPCR data normalization for gene expression analysis under salt stress were also identified.

Material and Methods

Plant material, stress imposition and sampling: Sixteen-month-old potted vines of grape variety 'Thompson Seedless' grown on its own root and grafted on '110R' rootstock were used for this experiment. Field capacity was calculated and 38 % moisture content was maintained by frequent irrigation. Salt treatment was imposed by irrigating vines with 150 mM NaCl salt solution. The control vines were irrigated with potable water (EC $\leq$ 0.7 dSm^{-1}). Young leaves and roots were sampled from control and treated vines at 6 h, 24 h, 48 h (early response), 7^{th} d, 15^{th} d (late response) after treatment. Samples were snap frozen in liquid nitrogen and stored at -80 °C till further use. Sample from single vine was considered as one biological replicate.

For expression analysis in different plant parts, buds, leaf, petiole, stem, flowers and young berries were collected from field grown grafted and own-root raised vines of 'Thompson Seedless'. Samples from single vines were pooled and considered as one biological replicate.

Identification of salt responsive target gene and primer design: 16,785 salt stress EST out of total > 350 thousand grape ESTs were downloaded from public domain database (www.ncbi.nlm.nih.gov) and processed. The process involved sequence cleaning, repeat masking, vector masking, organelle masking and sequence assembly of the data using EGAssembler. Blast2GO (Conesa and Götz 2008) was employed for their annotation. For the filtered unknown proteins, Phytozome 8.0, a comparative platform for green plant genomics was used to BLAT against *Vitis vinifera* followed by FGENESH gene finder tool to identify the genes. The length of contigs was extrapolated on left and right in search of gene. The *cis* regulatory elements in the promoter region of the candidate genes were obtained from PLACE database and Plant Transcription Factor Database (PlnTFDB) V 2.0 were further used to find the transcription factors present in assembled EST data. InterproScan and PANTHER were employed for motif identification.

Sequence analysis of *VviERF073*: Reference genome sequence of *Vitis* available at NCBI was used to design sequencing primers for the region covering entire coding region of *VviERF073*. The total genomic DNA from 'Thompson Seedless' was used for PCR amplification using Phusion HI-Fidelity DNA Polymerase (NEB Inc., USA). The PCR products were purified and sequenced using ABI big dye chemistry 3.1 (Applied biosystems). The sequence of all PCR products were aligned and aligned sequence was blasted against *Vitis* genome sequence. The complete sequence was used for gene prediction using FGENESH (Solovyev *et al.* 2006).

The predicted protein was used for homology search using blastp. A phylogenetic analysis of the *VviERF073* and various heterologous AP2/ERF genes was performed using MEGA 6 software (Tamura *et al.* 2013) and a phylogenetic tree was constructed using Neighbour joining method. ERF domain of *VviERF073* and heterologous genes was aligned using Clustal W (Larkin *et al.* 2007). Secondary structure of ERF domain was predicted using Quick2D (http://toolkit.tuebingen.mpg.de/quick2_d). Interaction of *VviERF073* with other proteins was analyzed using STRING V9.1 (Franceschini *et al.* 2013).

RNA extraction and first strand cDNA synthesis: 70-100 mg of frozen leaves were used for RNA extraction using Spectrum™ Plant total RNA kit (Sigma – Aldrich, USA) following manufacturer instructions. On-column DNase digestion was performed before RNA elution. For RNA extraction from roots, the method described by Tattersall *et al.* (2005) was used. Three biological replicates for each time point were used for RNA extraction. RNA concentration was measured by recording absorbance at A260 using a nano-spectrophotometer (Implen, Germany) and absorbance ratio A260/A280 (> 1.8) and A260/A230 (> 2.0) were used for estimating its purity. The RNA integrity was tested by electrophoresis on 1X TBE agarose gel.

One microgram of total RNA was used for cDNA synthesis using Superscript III (Invitrogen) as per the manufacturer's instructions. The synthesized first strand cDNA was diluted to 200 µL with RNase free water, mixed thoroughly, aliquoted and stored at -20 °C till use.

Primer designing, Real time PCR and data acquisition: Specific primers for *VviERF073* were designed using web based software Primer3Plus (Untergasser *et al.* 2007). The specificity of primer pair was confirmed by the presence of a single peak in melt curve analysis and no primer-dimer peaks in no-template-control

(NTC) reactions. The primer sequence and other details are given in supplementary Tab. 1. The qPCR reaction in 20 µL contained 3 µL of diluted cDNA, 200 nM each primer (forward and reverse) and 1 x Absolute qPCR SYBR Green Mix (Thermo Scientific, USA). No-template controls were included for each primer pair. Liquid handling system QIAgility 2000 (Qiagen, USA) was used for setting up qPCR reactions. Aliquots from same cDNA sample were used with all primer pairs. Reactions were performed on three biological replicates in duplicate. Real time PCR was performed in Rotor-Gene Q (Qiagen, Valencia, CA, USA) with the following temperature profile: initial denaturation at 95 °C for 15 min, 45 cycles of step 1, 95 °C for 20 s; step 2, 60 °C for 30 s and step 3, 72 °C for 30 s, hold at 72 °C for 2 min. At the end, a melt was performed at 50-99 °C with increase of 1 °C at each step, held 90 s for first step and 5 s for each step afterwards.

Rotor-Gene Q software ver 2.3.1 was used for the data acquisition and analysis. The amplification plots were analyzed with default threshold value and amplification cycles (Cq) were obtained. Five point serial dilutions of one of the cDNA in triplicate were used to generate standard curve. The slopes of the standard curve were used to determine PCR efficiency and correlation coefficient for each primer pair. The reaction efficiency was calculated using the equation $E = 10^{[-1/slope]} - 1$, where E is the reaction efficiency and slope is the gradient of the best fit line in the linear regression.

Identification of stable reference gene for qPCR data normalization: Eight candidate reference genes *i.e. GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase), *Actin*, *α-Tubulin*, *SAND* (SAND family protein), *EF1α* (Elongation factor 1-α), *SuTra* (Sucrose transporter), *AqPo* (Aquaporin) reported by Reid *et al.* (2006) and *UBI* (Fujita *et al.* 2005) were evaluated for their expression stability under salt stress in own rooted as well as grafted vines of 'Thompson Seedless'.

The expression stability of eight candidate reference genes was estimated by geNorm (Vandesompele *et al.* 2002) and NormFinder (Andersen *et al.* 2004) methods. The optimal number of reference genes for data normalization was estimated as described by Vandesompele *et al.* 2002.

Expression analysis of *VviERF073*: Relative expression of target gene *VviERF073* in treated vines at different time points was estimated using REST2009 (Pfaffl *et al.* 2004). Iteration was set to 10000 for the analysis. A pair-wise fixed reallocation randomization test is used to determine whether the difference in expression is significant. Two most stable reference genes based on geNorm ranking were used for the data normalization. Expression of *VviERF073* in different plant tissues was estimated by $2^{-\Delta C_T}$ method (Livak and Schmittgen 2001).

Results and Discussion

Identification, sequence and phylogenetic analysis of *VviERF073*: *In silico* analysis of 16,785 salt stress ESTs of *Vitis vinifera* identified gene bank accession XM_002272428 as one of the candidate salt stress responsive transcription factor and was selected for detailed analysis in variety 'Thompson Seedless'. This gene is located on chromosome 7.

A sequence of 1166 bp was obtained for 'Thompson Seedless' which included 39 bp 5' region, coding region and 3' region. FGENESH predicted a protein of 316 amino acids in this sequence. The predicted protein contained two exons and one intron. The amino acid sequence of predicted protein contains a single AP2/ ERF conserved domain (Supplementary Fig. 1). The protein had 100 % homology with grape predicted protein ERF073 (XP_002272464.1). We named this gene as *VviERF073*. The sequence information of *VviERF073* has been submitted to NCBI genebank (Gene bank ID KP256369).

NCBI BLAST analysis of this protein showed high homology with ERF genes from several other perennial fruit species. VviERF073 protein showed 53 % and 54 % homology respectively with ERF071 and ERF073 proteins of *Malus domestica.* Similarly, homology with several ERF proteins of *Prunus* was above 50 %. Amino acid sequence of VviERF073 had more than 50 % homology with ERF protein of other tree species like *Populus.* The conserved AP2/ ERF region of VviERF073 had more than 90 % homology with conserved domain of the AP2/ERF family of proteins of a large number of plant species. Seven conserved amino acid residues *i.e.* Gly-4, Arg-6, Glu-16, Trp-27, Leu-28, Gly-29 and Ala-38, characteristic of ERF family proteins in *Arabidopsis* (Nakano *et al.* 2006) were also present in VviERF073. The 14th and 19th amino acid residues in conserved domain, which determine the DNA binding specificity, were alanine and aspartic acid respectively.

A phylogenetic analysis of VviERF073 with heterologous ERF proteins of *Arabidopsis*, wheat, soybean, tomato was performed using MEGA6 (Tamura *et al.* 2013). ERF proteins representing all the subfamilies B1-B6 of *Arabidopsis* (Nakano *et al.* 2006) were included in the analysis. Phylogenetic tree (Supplementary Fig. 2) showed that VviERF073 is a B2 subfamily gene within ERF family. Analysis showed close relation of VviERF073 with *Arabidopsis* ERF proteins AtRAP2.3, AtRAP2.12, AtRAP2.3, soybean ERF proteins GmERF3 and GmERF7, wheat protein TaERF1 and tomato ERF proteins LeJERF1 and LeJERF3. The AP2/ ERF domain of VviERF073 was compared with related B2 proteins and its secondary structure was analyzed. ERF domain of these proteins formed three β-sheets and one α-helix (Supplementary Fig. 3).

Sequence analysis confirmed that the selected gene *VviERF073* belonged to ERF family of AP2/ERF class of plant specific transcription factors. ERF family is further subdivided into DREB and ERF main groups (Sakuma *et al.* 2002). Peptide analysis of VviERF073 revealed that conserved amino acid at 14 and 19 residues are specific to ERF group and not DREB/CBF group. AP2/ERF transcription factors are known to play a pivotal role in plant growth, development and abiotic stress response. The ERF family is considered to be the largest among AP2/ERF transcription factors (Nakano *et al.* 2006). Several studies have demonstrated the role of transcription factors of ERF family in salt stress acclimation in many plant species (Ayarpadikannan *et al.* 2014, Dong *et al.* 2012, Zhai *et al.* 2013) either as a modulator of transcription or a component of signaling path-

way. Overexpression of ERF genes such as *JERF3* (Wang *et al.* 2004) and *Sl-ERF.B.3* (Klay *et al.* 2014) in tomato, *Tsi* and *OPB1* in tobacco (Park *et al.* 2001) and *AtERF71/HRE2* in *Arabidopsis* (Park *et al.* 2011) conferred tolerance to salt in transgenic plants. Similarly, overexpression of ERF genes in tomato and Chinese cabbage improved tolerance to drought and salinity of transgenic plants (Lu *et al.* 2011).

In *Arabidopsis*, ERF group is further classified into six subfamilies B1-B6 (Sakuma *et al.* 2002). In phylogeny analysis, *VviERF073* grouped with ERF of other plant species belonging to B2 subfamily. Transcription factors belonging to B2 group are characterized by the presence of N-terminal MCGGAII/L sequence. Deletion studies in wheat (Xu *et al.* 2007) and tomato (Tournier *et al.* 2003) indicated that N-terminal sequence is required neither for nuclear/subcellular localization nor for transcriptional activity. However, its role in protein kinase interaction has been demonstrated (Xu *et al.* 2007).

Interaction of VviERF073 with other proteins: The probable interaction of this protein with other proteins was analyzed using String V9.1 (Franceschini *et al.* 2013). Analysis identified 16 different proteins which are likely to interact with VviERF073 (Supplementary Fig. 4). Majority of these proteins have not been assigned a function in *Vitis*, hence the role of interacting proteins was determined based on their homology to *Arabidopsis thaliana* (Supplementary Tab. 1). VviERF073 was predicted to interact with RD22-B (responsive to dehydration 22) protein, CPR5, a regulator of expression of PR genes, OPR2, an enzyme involved in jasmonic acid synthesis, several hormone receptors, enzymes involved in hormone metabolism, auxin binding and inducible transcription factor.

Plant hormones such as salicylic acid, jasmonic acid ethylene and ABA have been found to be involved in signaling crosstalk under abiotic and biotic stress conditions (Fujita *et al.* 2006). ABA is involved in both, synergistic and antagonistic cross-talk between abiotic and biotic stress conditions (Asselbergh *et al.* 2008). In protein - protein interaction analysis, VviERF073 is predicted to interact with several proteins involved in hormone binding and response, thus suggesting that this gene may be a component of hormone mediated response to salinity stress in grapevine.

Identification of stable reference genes under salt stress conditions: Gene expression analysis using real-time PCR (RT-PCR) is widely used to understand several biological processes. Selection of stably expressed reference gene for data normalization is considered to be one of the most critical factors for success of RT-PCR based quantification. Normalization of RT- PCR data with multiple internal control genes validated for their expression stability is recommended (Gutierrez *et al.* 2008). In recent years, identification of stable reference genes has been reported in a wide range of experimental conditions and/or tissue types for several plant species. In grape, stable reference genes for different berry stages (González-Agüero *et al.* 2013, Reid *et al.* 2006), biotic stress (Monteiro *et al.* 2013) and abiotic stress (Coito *et al.* 2012) have been reported, however no report on stable reference genes under salt stress is available. In our experiment, amplification efficiency (E) of candidate reference genes ranged from 92 % (Actin) to 105 % (SAND). The correlation coefficient (r^2) of linear standard curve varied between 0.98-0.99 (Supplementary Tab. 2). The product of all the primers resulted in single peak in melt curve analysis. The box plot of Cq data (Supplementary Fig. 5) indicated that the variation in expression was more in leaf samples of grafted vines for most of the reference genes as compared to own root samples. Since rootstocks are known to influence gene expression in the scion variety, the stability of eight reference genes under salt stress was estimated separately for samples from own rooted vines and grafted vines (Supplementary Fig. 6).

In the set of own root samples, geNorm identified *Actin* (M = 0.050) and *Tubulin* (0.051) as the two most stable genes although the M value of other genes except *EF1α* was *on par* with top two genes. However, NormFinder ranked *Actin* and *Aquaporin* as the two most stable genes in this set. On the other hand, *GAPDH* and *EF1α* were ranked as the most stable genes in grafted samples both by geNorm and NormFinder method. This is in accordance with Monteiro *et al.* (2013), who reported different sets of stable genes in leaf tissue for biotic stress in two genotypes but *EF1α* as a common stable gene in different samples. On the contrary Selim *et al.* (2012) reported *EF1α* as one of the least stable genes in leaf under biotic stress. Such conflicting reports about the stability of reference genes further strengthen the case for standardization of reference genes for each experimental condition. *GAPDH* and *Actin/tubulin* were ranked as the most stable genes in all sample set by both the algorithms. Our results demonstrated the effect of rootstock on the stability of reference genes under salt stress. Considering this, appropriate reference genes should essentially be identified for expression analysis in different stock-scion combinations.

Use of two or more reference genes for qPCR data normalization has been suggested by several researchers (Tricarico *et al.* 2002). Estimate of pairwise variation (V) in geNorm is used to determine the optimal number of reference genes suitable for data normalization. The estimation of optimal number of reference genes (Supplementary Fig. 7) indicated that the use of two reference genes is ideal for data normalization in own root set. The pairwise variation V2/V3 (0.10) was below the recommended value of 0.15. In the set of grafted samples, V2/V3 (0.18) and V3/V4 (0.17) were above 0.15, the value below this mark was obtained with V4/V5 suggesting that the use of 4 reference gene may be needed for normalization of real time PCR data for this set. However, in grafted samples, the stability value of top ranked three genes was statistically *on par* whereas M value of fourth ranked genes was significantly different from top two ranked genes. The relative expression values of target gene *VviERF073* with two, three and four reference genes were statistically *on par* at 6 h, 48 h and 7 days (Fig. 1). At 24 h and 15 days expression values with three and four reference genes were *on par*. Based on this observation, we considered use of two or three most stable reference genes to be appropriate for data normalization even though the V2/V3 and V3/V4 are slightly more than the cutoff value. Higher V value has been considered for determining optimal number of reference genes by other workers (Silveira *et al.* 2009) for different tested data sets.

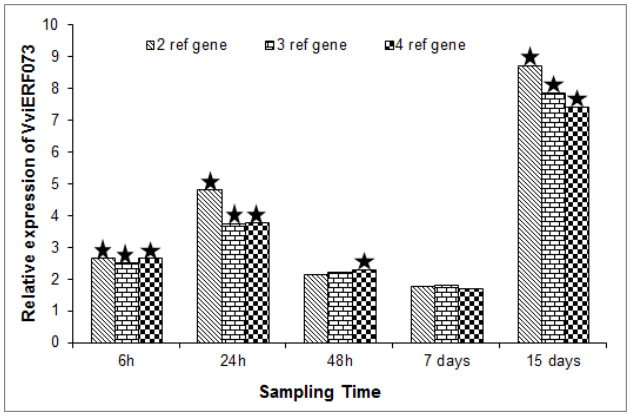

Fig. 1: Effect of number of reference genes on the relative expression of target gene *VviERF073* in the leaf of salt treated grafted vine. The qPCR data was normalized with two, three or four reference genes. ★ indicates that the difference in expression level in salt treated samples was statistically significant at $p < 0.05$ determined using REST software.

Expression of *VviERF073* in response to salinity stress: Expression of *VviERF073* was analyzed in response to salinity stress in leaves and roots. In leaf, the Cq value of *VviERF073* ranged between 18.37-23.92 with a mean value of 21.35 ± 1.50 in grafted vines whereas in own root samples, the mean Cq value of the *VviERF073* gene was 20.66 ± 0.62 and it ranged between 18.55 to 21.77. In roots of 'Thompson Seedless', the mean Cq value was 21.52 ± 1.28 and ranged between 19.06 to 24.68 whereas in roots of '110R' rootstock, the Cq value ranged between 20.51-31.31 with a mean value of 23.25 ± 2.74. The relative expression of *VviERF073* was estimated in leaves and roots of salt stressed grapevines and the results are presented in Fig. 2.

Effect of rootstock on the expression of *VviERF073* under salt stress: In leaves of 'Thompson Seedless' vines grafted on rootstock '110R', the relative expression of *VviERF073* increased in salt stressed samples at all the time points. There was more than two fold (2.7 fold) increase in its expression as early as 6 h of salt stress, which increased further at 24 h (4.8 fold). The expression levels in treated samples remained high at 48 h (2.1 fold) and 7 d (1.7 fold) as compared to control vines, although this increase was found to be statistically non-significant. At 15 d, there was a sharp increase in relative expression of *VviERF073* and its expression increased 8.7 fold in leaves of salt stressed vines. On the other hand, in leaves of grapevines with own root system, the relative expression of *VviERF073* did not change significantly at 6 h (1.2 fold) and 24 h (0.85). However, expression decreased at 48 h (0.76) which was statistically significant. The expression at 7 d (0.94) was not different from control samples. The expression level decreased further at 15 h (0.66) and was the least among all the time points.

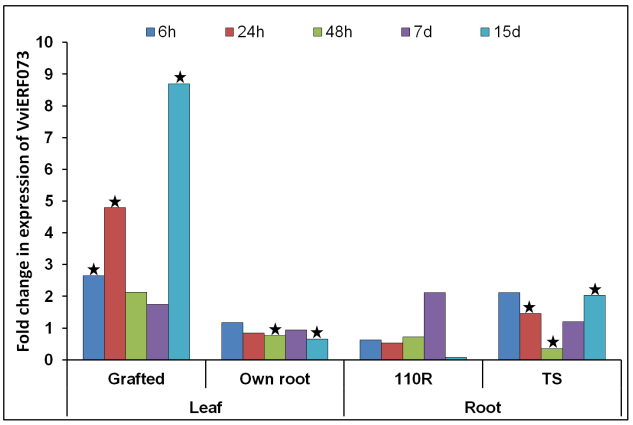

Fig. 2: Effect of salt stress on the expression of *VviERF073* in leaves and roots of grafted and own-root grapevines. The bar illustrates the fold change in expression in treated vines versus control vines. ★ indicates that the difference in expression level in treated vines was statistically significant at $p < 0.05$ determined using REST software.

Organ and genotype specific expression of *VviERF073* under salt stress: The expression analysis of this gene in roots of 'Thompson Seedless' and '110R' indicated organ and genotype specific expression pattern. In roots of 'Thompson Seedless', the expression varied at different time points. The expression in roots of treated vines was 2.11, 1.5, 0.36, 1.23- and 2.15-fold of control at 6 h, 24 h, 48 h, 7 d and 15 d respectively. The expression was significantly up-regulated at 24 h and 15 d, while down regulated at 48 h. Contrary to this, expression of *VviERF073* in the roots of '110R' did not change in response to salt stress.

Expression of *VviERF073* in different tissues: The expression of this gene was analyzed in vegetative (leaf, petiole, buds, stem) and reproductive (flowers and young berries (4-6 MM) tissues of field grown vines of 'Thompson Seedless' raised on own root as well as grafted on '110R'. The expression was estimated as relative to reference genes. The data in Fig. 3 indicated that this gene is expressed in all the tissues of grapevine although its expression pattern varied between own root and grafted vines. While in own rooted vines, highest expression was in reproductive tissues (flower and berries), the expression was the highest in the leaf of grafted vines. These results are in accordance with Licausi *et al.* (2010) who reported variable expression of this gene in different vegetative and reproductive tissues of grapevine cv. 'Corvina'.

In our analysis, expression of *VviERF073*, which was selected based on the analysis of salt stress EST libraries, increased rapidly several folds in the leaves of salt stressed vines. The increase was observed as early as six hours after imposition of salinity stress with further increase at 24 h. The expression of this gene in many tissues of field grown vines suggests its role in developmental processes, however, increased expression under salt stress indicated that this gene may have a positive function in salt stress response. Early

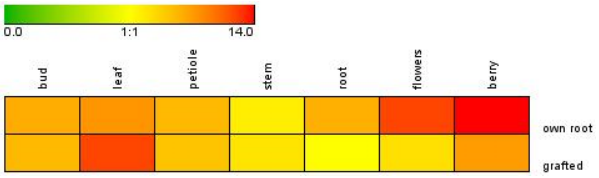

Fig. 3: Heat map of expression of *VviERF073* in different parts of grafted and own-root raised vines of 'Thompson Seedless'. The expression was estimated relative to two reference genes using $2^{-\Delta C_T}$ method.

and rapid increase in the expression of salt-responsive ERF has been reported in peanut (Chen *et al.* 2012) and wheat (Dong *et al.* 2012). ERF TFs of B2 group are shown to be responsive to multiple stresses including salt stress in wheat (Xu *et al.* 2007), soybean (Zhai *et al.* 2013) and other plant species. The change in expression in leaves in response to salt stress was substantial as compared to roots, suggesting organ specificity of the gene.

Rootstocks are widely used in fruit crops mainly to provide resistance to soil-borne pests and diseases, to improve fruit quality, to increase tolerance to abiotic stresses and to improve crop productivity (Gregory *et al.* 2013). Rootstocks are known to affect yield, productivity, quality and various physiological processes in the grape scion variety (Corso and Bonghi 2014, Marguerit *et al.* 2012). Salt tolerant grape rootstocks prevent uptake of Na^+ and Cl^- and translocation to other parts in the plant. Different rootstocks differ in their ability to exclude salts and response of the scion to soil salt varies according to the exclusion capability of the rootstock genotype (Fisarakis *et al.* 2001). Differential activation of genes of different pathways as affected by rootstock has been reported by several workers. Extensive transcriptional reprogramming in shoot apical meristem of grafted grapevines was reported by Cookson and Ollat (2013). Marè *et al.* (2013) also observed influence of soil and rootstock on the transcriptome of leaves of scion and differential activation of genes related to phenylpropanoid pathway, carbohydrate and energy pathways as well as stress response mechanism. In our analysis, also rootstock was found to influence the expression of *VviERF073* as differential expression of this gene was observed in the leaf of grafted and own root vines. This transcription factor gene, as a part of plant defense system, may be involved in rootstock mediated changes in other developmental processes in grafted vines which are better adapted to salt stress as compared to own root vines.

Conclusions

Transcription factor *VviERF073*, selected based on *in silico* analysis of EST database, was differentially expressed in response to salt stress. Varying level of expression was observed in two stock-scion combinations as well as two types of tissues *i.e.* leaf and root. The extent of its role in imparting salt tolerance at plant level needs further investigation. Analysis of stability of different candidate genes identified not only the stable reference gene under salt stress but also stock-scion combination specific stable reference genes. Based on our analysis we recommend use of two or three reference genes for data normalization. This analysis helped us to estimate expression of target gene with higher level of confidence.

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