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ORIGINAL ARTICLE

# Gene expression analysis in drought tolerant and susceptible black pepper (*Piper nigrum* L.) in response to water deficit stress

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**Abstract** Drought or water deficit stress is one of the main environmental stresses affecting plants, resulting in reduced productivity and crop loss. Black pepper, a major spice cultivated across the globe, is drought sensitive and water stress often results in plant death. The present study compared the difference in physiological parameters: relative water content (RWC) and cell membrane leakage, and also analyzed the differential expression of 11 drought responsive genes in drought tolerant and drought sensitive black pepper genotypes. Tolerant black pepper genotype exhibited significantly higher RWC and lower cell membrane leakage 10 days after stress induction than the sensitive genotype. The relative expressions of the 11 selected drought responsive genes were normalized against ubiquitin and RNA-binding protein which was identified as the most stable reference genes in black pepper under the present experimental condition using the RefFinder software. Dehydrin showed the highest transcript accumulation in both the black pepper genotypes under drought stress condition and the relative expression of the gene was higher in the tolerant genotype compared to the susceptible. Similar pattern of higher relative expression was also observed in the stress responsive gene, osmotin. The membrane protein aquaporin and the transcription factor *bZIP* were relatively down-regulated in the tolerant genotype. The differential expression of these important drought responsive genes in tolerant genotype of black pepper indicates its further usefulness in developing varieties with improved water stress tolerance.

**Keywords** Black pepper · Relative water content · Drought · Dehydrin · Osmotin · Aquaporin · Gene expression · Real time PCR

## Introduction

The king of spices, black pepper (*Piper nigrum*), is the most widely used spice around the world which has a major economic value. Rainfall has a key role in the production and cultivation of black pepper, but the climatic changes and erratic rainfall leading to limited water availability become a major constraint (Kandiannan et al. 2014). Dry spell, even for a very short duration, after the onset of flowering can be critical and leads to lower berry production in black pepper (Pillay et al. 1988). More than 1000 germplasm accessions of black pepper were screened at ICAR, Indian Institute of Spices Research, Kozhikode, and 20 drought tolerant accessions were identified. Of the identified accessions, a few are being employed to develop drought tolerant black pepper varieties.

The growth and productivity of plants are severely affected by environmental factors like drought and salt stress (Shao et al. 2009). Plants respond to water stress or desiccation at the biochemical, physiological, cellular, and molecular levels (Shinozaki and Yamaguchi-Shinozaki 2007), and the physiological and metabolic processes are reprogrammed during drought (Park et al. 2012). Comparatively, higher RWC (Jamaux et al. 1997;

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Krishnamurthy and Saji 2000; Altinkut et al. 2001; Colom and Vazzana, 2003) and low membrane leakage (Bajji et al. 2001; Krishnamurthy et al. 1998) are associated with drought tolerant genotypes. This physiological variation is used to screen for drought tolerance in a number of plants species and in black pepper (Krishnamurthy et al. 1998).

Drought tolerance in plant is under the control of polygenes displaying medium-to-low heritability (Ashraf 2010). The genes involved in drought tolerance are either induced or repressed during water stress (Shinozaki et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2005; Bartels and Sunkars 2005). Genes induced during drought stress can be broadly classified into two; genes which functions in protecting cells and regulatory proteins like transcription factors (Lata and Prasad 2011). Osmotin (*OSM*) (Kumar et al. 2015), dehydrin (*DHN*) (Hanin et al. 2011), aquaporin (*AQUA*) (Li et al. 2015), betaine aldehyde dehydrogenase (*BADH*) (Zhang et al. 2011), mitogen-activated protein kinase (*MAPK*) (Li et al. 2012), and heat-shock protein (*HSP*) (Al-Whaibi 2011) are some of the drought responsive genes reported to be differentially expressed during water stress condition in rice, wheat, potato, spinach, and *Arabidopsis*. The transcription factors, namely, basic leucine zipper protein (*bZIP*) (Babitha et al. 2015), *NAC* transcription factor (Hong et al. 2016), *MYB* transcription factor (Baldoni et al. 2015), dehydration-responsive element-binding protein (*DREB*) (Lata and Prasad 2011), and apetala 2 (*AP2*) (Mawlong et al. 2015), are involved in the drought responsive mechanism in rice, tobacco, and *Arabidopsis,* and influence the drought tolerance in these plants. Though drought is a main abiotic stress affecting black pepper, the role and expression of these drought responsive genes during water stress have not been investigated.

Thus, the present study was performed to understand the difference in response of tolerant and susceptible black pepper genotypes to water stress at both physiological and molecular levels. The main objective of the study was to identify the important drought responsive genes and study their expression pattern in tolerant and susceptible genotypes during water deficit stress in black pepper. The difference in RWC and membrane leakage and the differential expression of major drought responsive genes and transcription factors in tolerant and susceptible genotypes were studied.

## Materials and methods

### Plant material and induction of water stress

Six-month-old rooted cuttings of black pepper genotype, Acc. 4216, identified as drought tolerant via screening trials at ICAR-IISR, Kozhikode, and high yielding drought sensitive black pepper variety, Sreekara were used for the study. The plants were maintained under controlled greenhouse condition and healthy plants with 4–5 nodes were selected. Three biological replicates each (control and treatment) of drought tolerant and susceptible black pepper genotypes were taken for the study.

Prior to the drought stress induction, the black pepper plants were transferred to the plant growth chamber (York Scientific) maintained at 27 °C, 70% humidity with 12 h photoperiod. The plants were watered to field capacity (20–22% soil moisture content) for 2 weeks. The susceptible and tolerant plants were then subjected to treatment (water stress) by withholding water for 10 days, while the control plants were watered.

### Analysis of physiological parameters

The leaf samples and soil from control and treatment were taken for the analysis of physiological parameters. The soil moisture content, cell membrane leakage, and relative water content (RWC) were calculated.

### Determination of soil moisture content (SMC)

50 g soil was collected from 0 to 30 cm soil depth from each replication and the soil moisture content was measured using moisture balance MOC-120H (Shimadzu).

### Determination of relative water content (RWC)

Relative water content was determined as per Molaei et al. (2012) with minor modification. For RWC determination, youngest fully mature leaves were collected from the plants and the leaves were cut into 0.5 cm × 0.5 cm square bits and fresh weight of 10 bits was measured, and their turgid weights (TW) were measured after placing the leaf bits in distilled water for 4 h at room temperature (27 °C). Next, the dry weight of the bits was measured by oven drying them at 55 °C for 48 h. RWC was determined as:

$$\text{RWC} = (\text{fresh weight} - \text{dry weight})/(\text{turgid weight} - \text{dry weight}) \times 100.$$

### Determination of membrane leakage

Membrane leakage was determined as per Molaei et al. (2012) with minor modification. For membrane leakage determination, about 20 0.5 cm × 0.5 cm square bits were washed thoroughly x and then immersed in 10 ml of distilled water at room temperature. After 24 h, the electrical conductivity of the solutions was measured. The tissues were heated in boiling water bath (100 °C) for 15 min and cooled to room temperature, and the electrical conductivity

of the solutions was measured once again. Membrane leakage was calculated using the formula:

$$\text{Membrane leakage} = (1 - C_1/C_2)] \times 100,$$

where, $C_1$ and $C_2$ represent conductivity values of the sample after 24 h and after boiling respectively.

### Statistical analysis

Three biological replicates each for control and treatment were used for physiological parameter testing. The statistical significance of the values was analysed using two-tailed *t* test in Microsoft Excel.

### RNA isolation and cDNA synthesis

For expression study, youngest fully mature leaf (third/-fourth leaf from the apex) was collected from three different biological replicates (control and treatment), pooled, immediately frozen in liquid nitrogen, and stored at −80 °C until processing. Total RNA was extracted from leaves of drought induced and control samples by the Trizol (Ambion) method following the manufacturer's protocol. The integrity of RNA was analysed on 1.2% agarose gel, and the RNA Intergrity Number (RIN) and concentration of the sample were analyzed on Agilent 2100 Bioanalyzer. DNA free kit (Ambion) was used to remove the genomic DNA. 1 µg of DNA free RNA was used for cDNA synthesis anchored by oligod$T_{18}$ primer using Revert-aid M-MLV Reverse transcriptase (Thermoscientific) in a total volume of 20 µl.

### Real-time PCR analysis

The real-time analysis was carried out in the Rotor gene Q apparatus (QIAGEN) using QuantiFast SYBR Green PCR master mix (QIAGEN). 25 ng of RNA equivalent cDNA was used for a 20 µl real-time PCR reaction with a final concentration of 0.5 µM forward and reverse primers and 1× SYBR green buffer. The two-step PCR condition with an activation step of 95 °C for 5 min, and 35 cycles of denaturation at 95 °C for 20 s, and combined annealing-extension at 60 °C for 30 s was carried out. Three technical replicates each of all the samples were taken for real-time PCR analysis. A six point dilution curve analysis of the primer pairs was performed for analyzing the PCR efficiency of the primer pair (Supplementary file 1). Melt curve analysis (65–95 °C) was performed to confirm the amplification of a single product. No Template Control (NTC) and No Reverse Transcriptase Control (NRT) were tested for each primer pair to rule out non-specific amplification and the presence of residual genomic DNA in the sample. The amplicon of each primer pair was run on the gel to confirm single product and further sequenced (Supplementary file 2). The specific primers for normalizing genes and drought responsive genes used for the study (Table 1) were designed using the Primer3Plus software (http://primer3plus.com/) using sequence information from *P. nigrum* transcriptome data (PRJNA314826) developed at ICAR-IISR (George et al. 2012).

### Identification of stable reference gene on water stress

Expression profile of seven commonly used candidate reference genes; actin (*ACT*), ubiquitin (*UBI*), polyubiquitin (*POLUBI*), RNA-binding protein (*RNABP*), elongation factor 1alpha (*EF1A*), glyceraldehyde 3 phosphate dehydrogenase (*GAPD*), and ASK interacting protein (*SKP1*) were analyzed in control and treatment samples of three different black pepper genotypes including two tolerant and one susceptible to identify most stable reference gene in the given experimental condition. GeNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and ΔCt method (Silver et al. 2006) incorporated in the RefFinder software (Xie et al. 2012b) were used to identify the most stable reference gene. The comprehensive ranking of the reference gene stability identified by the RefFinder software was used to choose the stable reference gene. The pairwise variation (V) between the genes was calculated as per Vandesompele et al. 2002 to identify the least number of reference genes to be taken for the error free normalization of the current experimental data.

### Relative expression study of drought responsive genes in tolerant and susceptible black pepper genotypes

The relative expressions of eleven candidate drought responsive genes including five transcription factors (Table 1) were analyzed by real-time PCR in susceptible and tolerant genotypes of black pepper. The sequences of the candidate genes used for the study were mined from *P. nigrum* transcriptome (George et al. 2012) based on homology approach. For normalizing the real-time data, the minimum number of most stable candidate reference gene with the $V_{n/n+1}$ value <0.15 was used. Threshold cycle ($C_q$) value was acquired by the Rotor Gene-Q software after manual adjusting of threshold and baseline. The relative expression (fold change) of the genes was calculated using the $\Delta\Delta C_t$ method (Schmittgen and Livak 2008) against the respective control plants and the geometric average of $C_q$ of the selected reference genes was taken for normalizing the data. The relative expression of the genes was expressed in $\log_2$ value of fold change and was considered differentially expressed when the relative $\log_2$ value was at least ±1.

**Table 1** Details of candidate reference genes and drought responsive genes used for the study

| Gene | Predicted function | Primer sequence (5′–3′) |
|---|---|---|
| Candidate reference gene | | |
| ASK-interacting protein (*SKP1*) | Protein binding | F: TTCTGCACATCGAGAACGAC |
| | | R: CCGCTAAGTCGACGAAGAAC |
| Ubiquitin (*UBI*) | Regulatory protein | F: CGTGGAGGAATGCAGATTTT |
| | | R: CCTAGAAACCACCACGGAGA |
| *Polyubiquitin* (*POLUBI*) | Regulatory protein | F: TCGCACAACATCCAACATTT |
| | | R: CGCGGATCTATTGCGTTATT |
| RNA-binding protein (*RNABP*) | Post-transcriptional modification of mRNA | F: ACCTTTTACGCTGGGTTCCT |
| | | R: GTCACCCACCACACCTCTCT |
| Actin (*ACT*) | Cytoskeleton structural protein | F: ACATCCGCTGGAAGGTGC |
| | | R: TCTGTATGGTAACATTGTG |
| Elongation factor 1A (*EFIA*) | Translation | F: AAAGGTGACGACCATTCCAG |
| | | R: TCCCATCTCAGGTTTTGAGG |
| Glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) | Glycolysis, glucogenesis | F: AGGAGCGAAGAAGGTGTTGA |
| | | R: CCCAAACTTTTGATCGAGGA |
| Drought responsive genes and transcription factors | | |
| Basic leucine zipper protein (*bZIP*) | Transcription factor | F: ACTCATGGTCTTCGGCATTC |
| | | R: ACCGGCGTGGTCTGTATATC |
| *NAC* transcription factor | Transcription factor | F: TGGGCTTTTGCTCTGTCTTT |
| | | R: CTCACCCTTCTTCCCATTGA |
| Aquaporin (*AQUA*) | Integral membrane proteins | F: TGAATCCTGCCGTGACATTA |
| | | R: CGTGTTGTGCCGTATGAGAC |
| Betaine aldehyde dehydrogenase (*BADH*) | Abiotic tolerance | F: TCAGTACGTACGACCCGTCA |
| | | R: AGAGATCATCGGTCCCACTG |
| Myeloblastosis oncogene (*MYB*) | Transcription factor | F: GTGGCCTCCAATAAAAGCAA |
| | | R: ATCCATTCATCCCACCAAAA |
| Dehydration-responsive element-binding protein (*DREB*) | Transcription factor | F: GCAACCGAATTTCTCCGATA |
| | | R: AGCACCGTTTCCTTTTCTGA |
| Mitogen-activated protein kinase (*MAPK*) | Phosphorylation | F: GCTGCCAATACAGCATCAGA |
| | | R: GAGCTGTTCACATCCAAGCA |
| Heat-shock protein (*HSP70*) | Stress responsive protein | F: GTCCCCCACGAGTAACTTGA |
| | | R: GCAGAAATTGGGAGATGAGC |
| Apetala 2(*AP2*) | Transcription factor | F: ATGAGCTCGAGGGTAGACGA |
| | | R: CTATACCGGCTTTCCCACCT |
| Osmotin (*OSM*) | Stress responsive gene | F: ACCGTGTTTAAGACCGACCA |
| | | R: ACCATTTCATGGGCAAAAGA |
| Dehydrin (*DHN*) | Drought responsive gene | F: AGCAGATCAGCTGGAAGGAA |
| | | R: ATCAGTGGCACATTGTTCA |

## Results

### Analysis of physiological parameters

After 10 days of water stress, the susceptible variety Sreekara started showing wilting symptoms, while the leaves of the tolerant genotype did not wilt. The soil moisture of both the genotypes reduced drastically at 10 days after stress induction (DASI) (Table 2). The leaf physiological parameters, i.e., RWC and membrane leakage, differed significantly between the tolerant and susceptible genotypes after 10 days of water stress. Significantly higher RWC and lower membrane leakage were recorded in the tolerant genotype Acc 4216 compared to the susceptible black pepper variety Sreekara at 10 DASI.

**Table 2** Physiological parameters analyzed from Acc 4216 and Sreekara plants subjected to drought stress

| | Relative water content (%) | | Cell membrane leakage (%) | | Soil moisture content (%) | |
|---|---|---|---|---|---|---|
| | Control | 10 DASI | Control | 10 DASI | Control | 10 DASI |
| Acc. 4216 | 95.4 | 81.3 | 5.1 | 8.6 | 20.1 | 9.0 |
| Sreekara | 93.7 | 65.4 | 5.7 | 16.8 | 20.6 | 8.8 |
| *t* test | NS | $P < 0.001$ | $P < 0.01$ | $P < 0.001$ | NS | NS |

### Identification of stable reference gene on water stress

*RNABP* was found to be the most stable reference gene in black pepper during the water deficit stress condition by three out of four algorithms: Genorm, ΔCt method, and NormFinder. *UBI* was ranked as the most stable candidate reference gene two algorithms: BestKeeper and GeNorm (Fig. 1). The comprehensive ranking of the candidate reference genes by the RefFinder identified *RNABP*, *UBI*, and *EF1A* as the three most stable genes under the given set of condition. The maximum GeNorm stability value, M value of the genes tested, was 0.536 much lower than the recommended high M value cutoff of 1.5 ascertaining the suitability either of the candidate reference genes to be used for the normalization of real-time data in black pepper during drought. The $V_{2/3}$ value of the reference genes was calculated to be 0.083 (Fig. 2), and hence, the two most stable reference genes, *RNABP* and *UBI,* were chosen as candidate reference genes for the normalization of the gene expression in our study.

### Differential expression of drought responsive genes in black pepper

The expression of the genes studied was effected by drought stress in both genotypes of black pepper (Fig. 3). The relative expression of seven candidate genes followed similar pattern in the two genotypes studied. The expression of *AP2, MYB, DHN*, *OSM*, *NAC,* and *DREB* genes was found to be up regulated and that of *AQUA* was down-regulated during water stress in both the genotypes of black pepper when compared to their respective controls. On contrast, the expression of *bZIP* and *HSP70* was up-regulated in susceptible genotype and down-regulated in the tolerant. While the expression of *MAPK* and *BADH* was not altered during drought stress in the current experimental condition; their expression level remained at par with control plants. Among the up-regulated genes, the relative expression of *DHN*, *OSM*, *NAC,* and *DREB* was higher in tolerant genotype. However, the relative expression of *MYB* was higher in susceptible genotype. Although the expression of *AP2* was up-regulated during drought in both the genotypes, tolerance related variation of the gene could not be observed. The expression of *AQUA* was down-regulated during stress in both the genotypes, with the tolerant genotype exhibiting lower expression.

## Discussion

Drought is one of the major abiotic stresses affecting plants which can result in limited growth and photosynthesis. The RWC and membrane leakage of drought induced black pepper leaves were analyzed, and we have observed that the drought tolerant genotype, Acc 4216, had higher RWC and lower membrane leakage compared to the susceptible genotype, Sreekara at 10 DASI. The higher RWC and lower membrane leakage is an indicator for drought tolerance in black pepper (Krishnamurthy et al. 1998). As these physiological parameters are multi-gene controlled traits, the variation in the traits between tolerant and susceptible black pepper genotypes may reflect the differential expression of important drought responsive genes at the molecular level. The relative expression study of differentially expressed genes can help in understanding the key genes and mechanism involved in drought tolerance in black pepper.

Real-time PCR is routinely used for the absolute and relative gene expression analysis in plant system. The quality of RNA and its integrity can affect the downstream processing and the expression study. A good-quality RNA results in more efficient cDNA synthesis and accurate expression profiling by real-time PCR (Fleige and Pfaffl 2006). The accuracy and the validity of the real-time data depend on the reference gene used for the data normalization. Many reference genes are usually used for real-time data normalization without proper validation for its stability under the studied experimental condition. This can result in the erroneous and incorrect conclusion (Artico et al. 2010). Hence, every experiment needs the identification of the most stable reference gene for valid data normalization. Moreover, the use of more than one stable reference gene with low pair wise variation can give accurate data normalization (Vandesompele et al. 2002). The $V_{2/3}$ value calculated was 0.083 (<0.15 threshold, Vandesompele et al. 2002), and hence, two genes are enough to accurately normalize the real time expression data in black pepper under drought stress. Therefore,

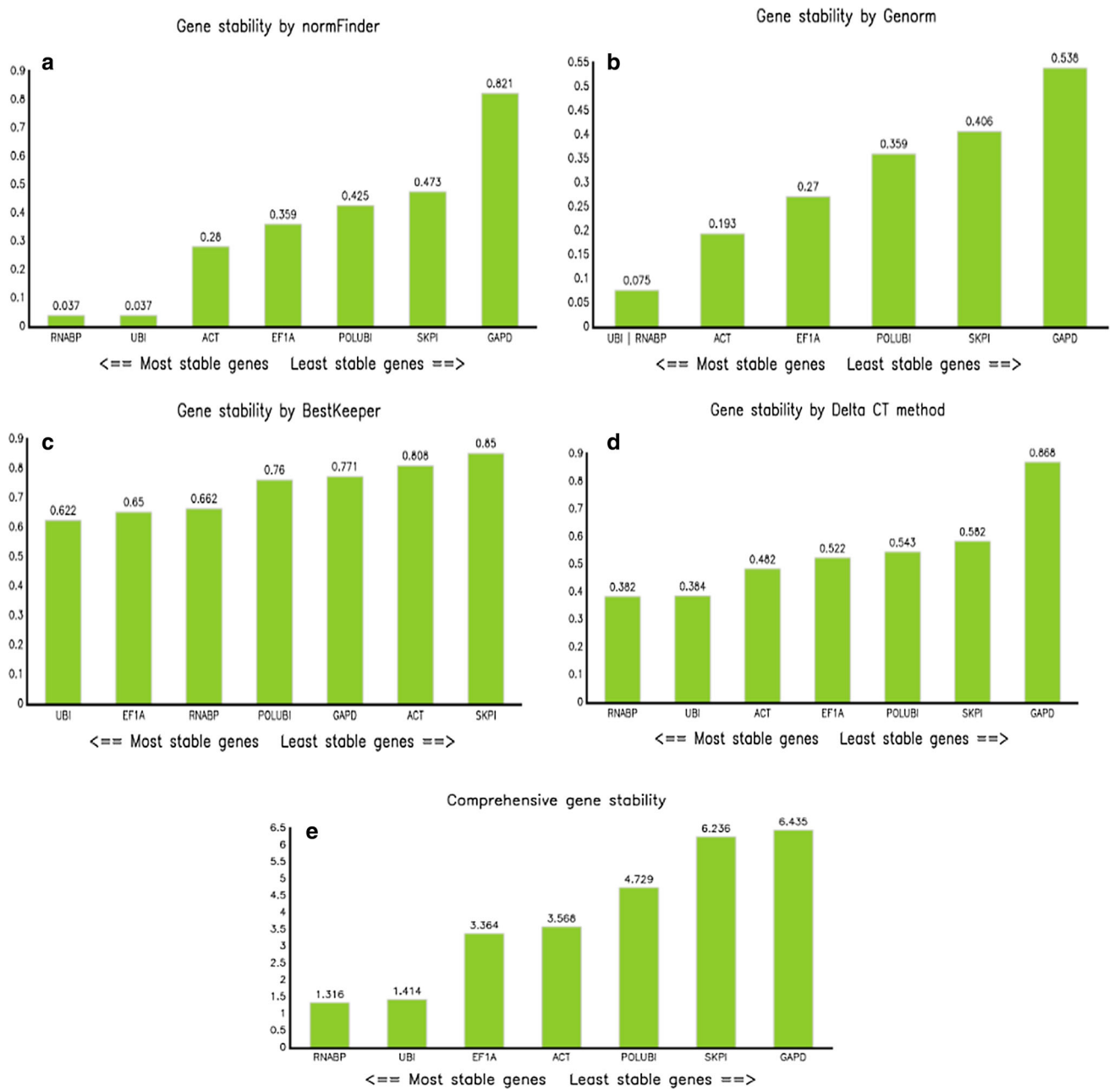


**Fig. 1** Identification of the most stable reference gene in black pepper under drought stress. **a** NormFinder, **b** GeNorm, **c** BestKeeper, **d** $\Delta C_t$ method, and **e** comprehensive gene stability by RefFinder

*RNABP* and *UBI* found to be the most stable candidate reference gene were selected for the data normalisation.

The relative quantification analysis of 11 genes identified the differential transcript profile in the tolerant and the susceptible plants. Two important genes associated with drought tolerance in plants, viz, *DHN* and *OSM,* were significantly up-regulated in both the pepper genotypes when subjected to drought stress with a relatively higher transcript accumulation in tolerant genotype. *AQUA* and *bZIP* genes were down-regulated in drought tolerant genotype of black pepper compared to the susceptible.

Dehydrin (*DHN*) is one of the most important genes expressed in plants during water deficit condition (Close 1996). These proteins of LEA family are expressed when the plant is subjected to abiotic stress, including water stress, cold stress, salt stress, and also during the late embryogenesis stage (Yang et al. 2012). Dehydrins help to maintain large amounts of water inside the plant cell during water stress, thereby protecting the plant proteins and

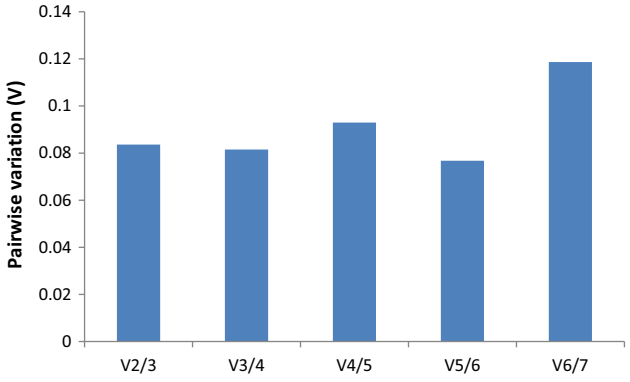


**Fig. 2** Pairwise variation analysis of seven candidate reference genes under drought stress

biomembranes (Tunnacliffe and Wise 2007; Battaglia et al. 2008). Elevated expression of dehydrin transcripts was reported during seed formation (Omar et al. 2013) and drought stress (Yang et al. 2012) in other plants. The over-expression of this gene is associated with drought tolerance in transgenic plants (Xie et al. 2012a). Drought tolerant lines of sunflower displayed a higher expression of dehydrin compared to the sensitive lines when subjected to progressive drought stress (Cellier et al. 1998). In the present study, tolerant genotype of black pepper showed a 473-fold ($\log_2$ 8.8) increase of dehydrin expression at 10 DASI. The water retention property of the dehydrin protein may contribute for the higher RWC in the tolerant genotype when subjected to water stress.

Osmotic adjustment (OA) is a very important mechanism in plants, whereby they try to overcome drought or salinity stress (Zhang et al. 1999). Osmotins are proteins that take part in the osmotic adjustment and are over-expressed in the plant tissue in response to salt or water stresses (Singh et al. 1987). During water stress condition, they either induce structural or metabolic changes in the plant cell or bring about the compartmentalization of solutes (Raghothama et al. 1993; Barthakur et al. 2001). Osmotin plays a role abiotic stress tolerance and up-regulation of the transcript is observed when the plants are subjected to either biotic or abiotic stresses (Zhu et al. 1995; Zhang and Shih 2007). Over-expression of *OSM* was associated with drought and salt tolerance in tomato and resulted in the improved RWC, chlorophyll, and proline content in the plant (Goel et al. 2010). Significant up-regulation of *OSM* gene was observed black pepper when subjected to drought stress, which corresponds with the previous reports in other plants (Parkhi et al. 2009; Goel et al. 2010; Subramanyam et al. 2011). Tolerant genotype of black pepper exhibiting higher *OSM* accumulation had improved RWC compared to the susceptible black pepper genotype exhibiting lower *OSM*, which is in conformity with the report in tomato (Goel et al. 2010).

Aquaporins are water channel proteins monitoring the movement of water across the membrane. Though higher expression of *AQUA* is often associated with higher drought tolerance in many plants, negative correlation of the gene with drought tolerant property is also reported. Over-expression of aquaporin in *Arabidopsis* resulted in the higher water loss, low RWC and water potential, and decreased drought resistance (Li et al. 2015). Similarly, transgenic tobacco over expressing the *AQUA* gene also showed faster wilting during water stress (Aharon et al. 2003). The negative regulation of the gene in the tolerant black pepper may contribute towards the reduced water transport out of the cell during drought stress and hence aid in the maintenance of water within, thus contributing to drought tolerance.

The up-regulation of the transcription factor *bZIP* is associated with drought tolerance in plants (Kang et al. 2002; Chen et al. 2012) and it functions by regulating the expression of the many drought responsive genes during

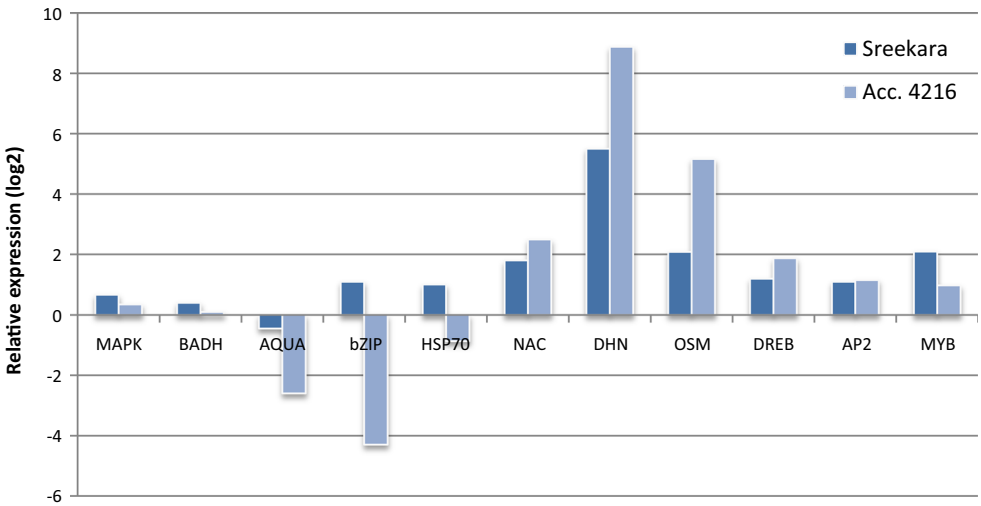


**Fig. 3** Expression of drought responsive genes in tolerant (Acc-4216) and susceptible (Sreekara) black pepper variety in response to drought stress

water deficit stress. In contrast, Liu et al. (2012) reported the down-regulation of many drought responsive genes including LEA gene in the transgenic rice over expressing *bZIP,* and have identified *bZIP* as a negative regulator of drought stress in rice. In the present study, the tolerant genotype showed down-regulation of the *bZIP* transcription factor, contrasting with the expression of drought responsive genes *DHN* and *OSM*. The down-regulation of the black pepper *bZIP* transcription factor and the simultaneous higher up-regulation of the important drought responsive genes in black pepper may suggest a similar role where *bZIP* act as a negative regulator in black pepper.

The short-term acclimation response of black pepper is investigated in the present study, where black pepper is subjected to 10 days of water withdrawal and the plants exhibit only initial signs of wilting. Long-term response of black pepper might involve the expression of different drought responsive genes at varying levels which may have to be further investigated.

## Conclusion

The tolerant and susceptible black pepper genotypes responded differently at the physiological and molecular levels when subjected to drought stress. The higher relative water content and lower membrane leakage were associated with the drought tolerance in black pepper. This corresponds to the effect of differential expression of genes in the tolerant and susceptible genotypes. The study identified a relatively higher expression of *DHN, OSM,* and down-regulation of *AQUA* and *bZIP* in drought tolerant genotype compared to the susceptible. *OSM* and *AQUA* genes may function together to maintain the cellular water content by adjusting the osmotic potential and by reducing the efflux of water through the membrane channels. At the same time, accumulation of *DHN* in tolerant genotype may also contribute towards the higher retention of water in leaves during drought. Drought tolerance in black pepper may be due to the combined effect of more than one gene that are induced or repressed during the stress. This study analyzed the specific drought-responsive genes in an attempt to understand the molecular mechanisms of the trait. The expression or repression of these genes may be very important for imparting water deficit tolerance in black pepper. It is also desirable to identify the promoters and single nucleotide polymorphisms of these genes for further elucidation of molecular mechanisms associated with water deficit stress tolerance.

**Author contribution statement** KJG designed the experiment; NM conducted the experiments and drafted the manuscript; IPVK helped in carrying out the research work. KSK carried out the physiological experiments. All authors have written relevant sections in the manuscript, and provided critical review and revision of the manuscript.

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## References

Aharon R, Shahak Y, Wininger S, Bendov R, Kapulnik Y, Galili G (2003) Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. Plant Cell 15:439–447

Altinkut A, Kazan K, Ipekci Z, Gozukirmizi N (2001) Tolerance to paraquat is correlated with the traits associated with water stress tolerance in segregating F2 populations of barley and wheat. Euphytica 121:8186

Al-Whaibi MH (2011) Plant heat-shock proteins: a mini review. J King Saud Univ Sci 23:139–150

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250

Artico S, Nardeli SM, Brilhante O, Fátima MG, Alves-Ferreira M (2010) Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data. BMC Plant Biol 10:49

Ashraf M (2010) Inducing drought tolerance in plants: recent advances. Biotechnol Adv 28:169–183

Babitha KC, Ramu SV, Nataraja KN et al (2015) *EcbZIP60*, a basic leucine zipper transcription factor from *Eleusine coracana* L. improves abiotic stress tolerance in tobacco by activating unfolded protein response pathway. Mol Breed 35:181

Bajji M, Kinet JM, Lutts S (2001) The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regul 36:61–70

Baldoni E, Genga A, Cominelli E (2015) Plant MYB transcription factors: their role in drought response mechanisms. Int J Mol Sci 16(7):15811–15851

Bartels D, Sunkars R (2005) Drought and salt tolerance in plants. CRC Crit Rev Plant Sci 24:23–58

Barthakur S, Babu V, Bansal KC (2001) Over expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. J Plant Biochem Biotechnol 10:31–37

Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Campos F, Covarrubias AA (2008) The enigmatic LEA proteins and other hydrophilins. Plant Physiol 148:6–24

Cellier F, Conejero G, Breitler JC, Casse F (1998) Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower. Plant Physiol 116:319–328

Chen H, Chen W, Zhou J, He H, Chen L, Chen H et al (2012) Basic leucine zipper transcription factor OsbZIP16 positively regulates drought resistance in rice. Plant Sci 193–194:8–17

Close TJ (1996) Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. Plant Physiol 97:795–803

Colom MR, Vazzana C (2003) Photosynthesis and PSII functionality of drought-resistant and droughtsensitive weeping lovegrass plants. Environ Exp Bot 49:135–144

Fleige S, Pfaffl MW (2006) RNA intergrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 27:126–139

George JK, Kumar VIP, Anandaraj M (2012) Transcriptomics approaches for gene discovery in plants—a case study in Piper. Agrotechnology 1:2

Goel D, Singh AK, Yadav V, Babbar SB, Bansal KC (2010) Overexpression of osmotin gene confers tolerance to salt and drought stresses in transgenic tomato (*Solanum lycopersicum* L). Protoplasma 245:133–141

Hanin M, Brini F, Ebel C, Toda Y, Takeda S, Masmoudi K (2011) Plant dehydrins and stress tolerance. Plant Signal Behav 6:1503–1509

Hong Y, Zhang H, Huang L, Li D, Song F (2016) Overexpression of a stress-responsive NAC transcription factor gene ONAC022 improves drought and salt tolerance in rice. Front Plant Sci 7:4

Jamaux I, Steinmertz A, Belhassen E (1997) Looking for molecular and physiological markers of osmotic adjustment in sunflower. New Phytol 137:117–127

Kandiannan K, Krishnamurthy KS, Anke Gowda SJ, Anandaraj M (2014) Climate change and black pepper production. Indian J Arecanut Spices Med Plants 16:31–37

Kang J, Choi H, Im M, Kim SY (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell 14:343–357

Krishnamurthy KS, Saji KV (2000) Response of piper species to water stress. Indian J Hortic 63:433–438

Krishnamurthy KS, Ankegowda SJ, George JK (1998) Impact of water stress on some physiological parameters in black pepper. In: Sadanandan AK, Krishnamurthy KS, Kandiannan K, Korikanthimath VS (eds) Water and nutrient management for sustainable production and quality of spices. Indian Society for Spices, Calicut, pp 153–157

Kumar SA, Kumari PH, Kumar GS, Mohanalatha C, Kishor PBK (2015) Osmotin: a plant sentinel and a possible agonist of mammalian adiponectin. Front Plant Sci 6:163

Lata C, Prasad M (2011) Role of DREBs in regulation of abiotic stress responses in plants. J Exp Bot 62:4731–4748

Li Y, Feng D, Zhang D, Su J, Zhang Y, Li Z, Mu P, Liu B, Wang H, Wang J (2012) Rice MAPK phosphatase IBR5 negatively regulates drought stress tolerance in transgenic *Nicotiana tabacum*. Plant Sci 10:188–189

Li J, Ban L, Wen H, Wang Z, Dzyubenko N, Chapurin V, Gao H, Wang X (2015) An aquaporin protein is associated with drought stress tolerance. Biochem Biophys Res Commun 459:208–213

Liu C, Wu Y, Wang X (2012) bZIP transcription factor OsbZIP52/RISBZ5: a potential negative regulator of cold and drought stress response in rice. Planta 235:1157–1169

Mawlong I, Ali K, Srinivasan R et al (2015) Functional validation of a drought-responsive AP2/ERF family transcription factor-encoding gene from rice in *Arabidopsis*. Mol Breed 35:163

Molaei P, Ebadi A, Namvar A, Khandan Bejandi T (2012) Water relation, solute accumulation and cell membrane injury in sesame (*Sesamum indicum* L.) cultivars subjected to water stress. Ann Biol Res 3:1833–1838

Omar SA, Elsheery NI, Kalaji HM, Ebrahim MKH, Pietkiewicz S, Lee CH, Allakhverdiev SI, Xu ZF (2013) Identification and differential expression of two dehydrin cDNAs during maturation of Jatropha curcas seeds. Biochemistry (Moscow) 78(5):485–495

Park W, Scheffler BE, Bauer PJ, Campbell BT (2012) Genome-wide identification of differentially expressed genes under water deficit stress in upland cotton (*Gossypium hirsutum* L.). BMC Plant Biol 12:90

Parkhi V, Kumar V, Sunilkumar G, Campbell LM, Singh NK (2009) Expression of apoplastically secreted tobacco Osmotin in cotton confers drought tolerance. Mol Breed 23:625–639

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515

Pillay VS, Sasikumaran S, Ibrahim KK (1988) Effect of rainfall pattern on the yield of black pepper. In: Agrometeorology of plantation crops (Pl. 152–159). Kerala Agricultural University, Trichur

Raghothama KG, Liu D, Nelson DE, Hasegawa PM, Bressan RA (1993) Analysis of an osmotically regulated pathogenesis-related osmotin gene promoter. Plant Mol Biol 23:1117–1128

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108

Shao HB, Chu LY, Jaleel CA, Manivannan P, Panneerselvam R, Shao MA (2009) Understanding water deficit stress-induced changes in the basic metabolism of higher plants-biotechnologically and sustainably improving agriculture and the eco-environment in arid regions of the globe. Crit Rev Biotechnol 29:131–151

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6:410–417

Silver N, Best S, Jiang J, Thein SL (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol 7:33

Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfankoch E, Regnier FE, Bressan RA (1987) Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol 85:529–536

Subramanyam K, Sailaja KV, Subramanyam K, Rao DM, Lakshmidevi K (2011) Ectopic expression of an Osmotin gene leads to enhanced salt tolerance in transgenic chilli pepper (*Capsicum annum* L). Plant Cell Tiss Organ Cult 105:181–192

Tunnacliffe A, Wise MJ (2007) The continuing conundrum of the LEA proteins. Naturwissenschaften 94:791–812

Vandesompele J, De Preter K, Pattyn F et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034

Xie C, Zhang R, Qu Y, Miao Z, Zhang Y, Shen X, Wang T, Dong J (2012a) Overexpression of MtCAS31 enhances drought tolerance in transgenic Arabidopsis by reducing stomatal density. New Phytol 195:124–135

Xie F, Xiao P, Chen D, Xu L, Zhang B (2012b) miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol 80:75–84

Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-acting regulatory elements in osmotic-and cold-stress-responsive promoters. Trends Plant Sci 10:88–94

Yang Y, He M, Zhu Z et al (2012) Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress. BMC Plant Biol 12:140

Zhang Y, Shih DS (2007) Isolation of an osmotin-like protein gene from strawberry and analysis of the response of this gene to abiotic stresses. J Plant Physiol 164:68–77

Zhang J, Nguyen HT, Blum A (1999) Genetic analysis of osmotic adjustment in crop plants. J Exp Bot 50(332):291–302

Zhang N, Si H, Wen G, Du H, Liu B, Wang D (2011) Enhanced drought and salinity tolerance in transgenic potato plants with a BADH gene from spinach. Plant Biotechnol Rep 5:71

Zhu B, Chen TH, Li PH (1995) Activation of two osmotin-like protein genes by abiotic stimuli and fungal pathogen in transgenic potato plants. Plant Physiol 108:929–937