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Analyses of leaf morphological changes and expressed genes related to water shortage of *Fagus orientalis* populations in a drought gradient

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Abstract

Climate change models predict an increase in mean annual temperature, a decrease in precipitation and consequently a scarcity of water resources during the growing season for Iran, including the northern forests of the country. One of the consequences is increased water shortage stress during the summer season. *Fagus orientalis* Lipsky is one of the most important commercial broad leaf species in the Hyrcanian forests and its potential of phenotypic and genetic adaptation to the climate change is essentially vital. The present research was carried out along two precipitation gradients from very humid to semi-humid conditions on four beech populations. The results of the flexibility of the leaf morphological traits showed that the populations of dryer regions had smaller and more elongated leaves than other populations. Evaluation of the expression of seven candidate genes in response to water shortage showed that the expression of aldehyde dehydrogenase, ascorbate peroxidase, Dehydrin (DHN), early responsive to dehydration, late embryogenesis abundant and Cys/His-Type Zinc-Finger in the eastern population was higher than that in the western population, while a difference in elevation gradient was only observed in the expression of some genes. Therefore, the natural selection pressure in the past evolutionary periods in the west-east gradient seems to be higher than the elevation gradient and has led to greater resistance to drought. Principal component analysis revealed that aldehyde dehydrogenase genes (ALDH), early responsive to dehydration (ERD), late embryogenesis abundant (LEA) and DHN are the most effective genes responding to water shortage stress for Oriental beech in the Hyrcanian forests.

Keywords: drought gradient, *Fagus orientalis*, gene expression, leaf morphology

Introduction

Oriental beech (*Fagus orientalis* Lipsky) is one of the most important ecological components of Hyrcanian forests and industrial wood production in northern Iran. This species is distributed from Gilan to Golestan provinces. The average annual rainfall in the Hyrcanian forests varies with an occasional record of 2,000 mm (Sagheb Talebi et al. 2014). According to the climatic data from the nearest meteorological stations, the average annual temperature in this region has varied from 15°C in the west to 17.5°C in the east over the past decade. The ecological dryness coefficient is negligible in the west regions and a dry season is absent in many parts. However, this value increases toward the east, reaching up to three months in Gorgan. Based on the research and assessment conducted during the climate change enabling activity project under the UNFCCC (United Nations Framework Convention on Climate Change) and using the scenarios proposed by the IPCC (Intergovernmental Panel on Climate Change), it is estimated that if the CO_2 concentration doubles by the year 2100, the average temperature in Iran will increase by 1.5–4.5°C, which will cause significant changes in abundance of water resources, energy demand, agricultural products and coastal zones (Amiri and Eslamian 2010). Availability of

water is one of the most important factors which determines geographical distribution and productivity of plants (Bartels 2001). Beech is a drought-sensitive species and further increase of drought periods will have a negative influence on its growth and competitive ability (Rennenberg et al. 2004, Betsch et al. 2011). Beech seedlings are most sensitive to drought stress. One study showed that in the following year after the severe drought in 2003, seedlings reacted with reduced growth (Czajkowski et al. 2005). In addition, dryer air alone is stressful for seedlings, reducing biomass production and leaf growth (Lendzion and Leuschner 2008). Several investigations showed that different beech provenances differ in their susceptibility to drought stress (Peuke et al. 2002, Schraml and Rennenberg 2002, Czajkowski and Bolte 2005). In general, provenances from dryer regions are better adapted to drought stress (Tognetti et al. 1995, Schraml and Rennenberg 2002). So, it is important to understand the drought adaptation potential of this species.

The phenotypic formation that occurs broadly in the leaf characteristics is recognized as a response to the environmental influences (McLellan 2000). The leaf morphological studies show that thick leaves are considered as a structure for resistance to wilt in arid, dry and sunny environments (Abrams 1990). In addition, studies show that large leaves will be more vulnerable to water shortage than smaller leaves (Warren et al. 2005). Most observations of the leaf morphological changes under water shortage conditions indicate a reduction in the leaf area and specific leaf area (Fonseca et al. 2000).

Drought is a multidimensional environmental factor, affecting tree responses from the molecular level to the forest stand level (Hamanishi and Campbell 2011). Drought triggers the production of abscisic acid (ABA), a phytohormone which in turn causes stomatal closure and induces expression of stress-related genes (Shinozaki et al. 2007). Several drought-inducible genes are induced by exogenous ABA treatment, whereas others are not affected. Drought stress often leads to the accumulation of reactive oxygen species (ROS). Plants have evolved a series of antioxidative systems, which are composed of metabolites such as ascorbate, glutathione, tocopherol, and enzymatic scavengers such as superoxide dismutase (SOD), peroxidase and catalase (Asada 1999) to keep the levels of active oxygen species under control. The antioxidative enzymes SOD and APX (ascorbate peroxidase) detoxify superoxide radicals and hydrogen peroxide, respectively. Their role in mediating drought tolerance has been known for a long time (Gupta et al. 1993, Mittler and Zilinskas 1994, Badawi et al. 2004).

To address the adaptation of *Fagus orientalis* in response to drought, we compared expression of seven key genes: aldehyde dehydrogenase (ALDH), ascorbate peroxidase (APX), isocitrate dehydrogenase, NADP-dependent ICDH (isocitrate dehydrogenase), Dehydrin (DHN), early responsive to dehydration (ERD), late embryogenesis abundant (LEA), Cys/His-Type Zinc-Finger Protein (ZFP) among the four beech populations along a drought gradient from north-west to north-east of Iran.

Aldehydes, which are formed as the result of stress-induced lipid peroxidation (Bartels and Sunkar 2005) are removed by ALDH (aldehyde dehydrogenase). Overexpression of ALDH increases dehydration tolerance in *Arabidopsis* (Sunkar et al. 2003). Most of the studied plant ALDH genes are shown to be induced under high salinity or water deficit conditions, suggesting possible roles of these genes in improving the plant osmotic stress tolerance (Kotchoni and Bartels 2003, Kirch et al. 2004, Kirch et al. 2005). ICDH may play an antioxidant role during oxidative stress and cytosolic ICDH may involve in the supply of NADPH (nicotinamide adenine dinucleotide phosphate) needed for plants against oxidative damage (Liu et al. 2010). Several studies have also suggested the potential ICDH regenerating function. Plants exposed to oxidative, abiotic stresses and trace metal ions, such as cadmium-treated or nickel-treated *Silene italica*, showed higher ICDH activity (Gálvez et al. 2005, Daniel et al. 2007). These results suggest that ICDH has a protective antioxidant role against certain environmental stresses in plants. Dehydrins (DHNs) are a class of hydrophilic thermostable stress proteins with a high number of charged amino acids that belong to the Group II Late Embryogenesis Abundant (LEA) family. Genes that encode these proteins are expressed during late embryogenesis, as well as in vegetative tissues subjected to drought, low temperature and high salt conditions (Nylander et al. 2001, Kim et al. 2010). The ERD (early response to dehydrin) genes are defined as those genes that are rapidly activated during drought stress. The encoded proteins show a great structural and functional diversity and constitute the first line of defence against drought stress in plants. ERD15 from Arabidopsis has been functionally characterized as a common regulator of the abscisic acid (ABA) response and salicylic acid (SA)-dependent defence pathway (Kariola et al. 2006). Drought-inducible genes display characteristic cis-acting elements such as DRE/CRT (dehydration-responsive element/C-repeat), ABRE (ABA responsive element), etc. Regulation of gene expression through DRE/CRT cis-elements appears to be mainly ABA-independent, whereas ABRE controlled gene expression is mainly ABA-dependent. In addition to these major pathways, other regulators, including the NAC, MYB/MYC, WRKY and Zn finger TF families also have important roles in response to abiotic stresses (Soren et al. 2012).

In the present study, we selected four beech populations along two precipitation gradients from very humid to semi-humid conditions (west, east and elevation extremes) and determined the phenotypic differences of seedlings in terms of the leaf morphology characteristics to assess the adaptation of the traits to drought conditions. Also, some natural regeneration from these populations was used in the experimental growth chamber with two soil moisture

levels to investigate the expression of genes related to ABA-signalling and stress. We hypothesized that populations from dryer regions exhibit constitutively higher expression levels of the stress-related genes and better leaf plasticity to water shortage. These indicators can be used to evaluate and improve other populations. Also, since there is no report on the sequencing of these candidate genes for Oriental beech, it was attempted to amplify and sequence the genes studied to determine their structural and functional similarity with the genes of other species through phylogenetic studies.

Materials and methods

Field sites

According to the distribution of beech in the northern forests of Iran, populations at the end of the eastern distribution in Golestan (Shastkalateh pop.) and western distribution in Gilan province (Shafarod pop.) as well as two populations at lower and upper levels of elevation (latitudinal) distribution in the middle of the west-east distribution in Mazandaran province (Kheyroud downland pop. and Kheyroud upland pop.) were selected (Figure 1, Table 1).

Leaf morphological study

The phenotypic differences of seedlings of different regions in terms of the leaf morphology characteristics were studied to assess the adaptation of the traits to drought conditions in the studied populations. For this purpose, leaves from 30 seedlings were sampled at a height of about 70 cm in each population with about 50 meters from each other. Morphological traits of leaves including the leaf length (LL), leaf width (LW), leaf perimeter (LP), leaf index (LI = LL / LW × 100), leaf shape factor (SF), leaf area (LA), petiole length (PL), distance between the lower point of the lamina and the maximum width point on the axis (LMW), the angle between the first sub and primary vein (AN), maximum width index (MWI = LMW / LL × 100), petiole index (PL = PL / LL × 100), Radius Coefficient (RC: radius area / radius based on the perimeter) and number of veins (NV) were measured, counted and calculated by Leaf Area Meter. Then, the difference between populations was investigated by the analysis of variance and Duncan's multiple comparison using the IBM SPSS Statistics software package, version 21.0 (IBM 2012).

Figure 1. Distribution of studied populations in the Hyrcanian forests

Gene expression analysis

Nine seedlings (height of ~0.5 m) from each population were sampled and transferred to the laboratory in a plastic pot. The experiment was executed in the growth chamber of the Biotechnology Laboratory of the Forest Science Faculty, located in the Gorgan University, Iran. The control and two water shortage stress levels, including 25% and 12.5% available water were treated to investigate the expression of genes related to ABA signalling (three seedlings per treatment). To apply water, the weight method was used. Once every day all pots were weighed using a precision portable balance device with an accuracy of ± 5 g. Then the moisture of the pots was set based on the soil moisture curve. This method is easily adapted for potted plants. AW (available water) was calculated using the following formula:

$$AW = FC - PWP,$$

where FC is the soil field capacity, and PWP is the soil permanent wilting point (Hosseini et al. 2015).

Water shortage treatment continued for three weeks in each level and then leaves were collected and frozen in liquid N_2 immediately and stored at –20°C before analysis. The response of seedlings under two soil water levels were compared among populations.

Isolation and determination of RNA quality and quantity

The RNA was extracted from approximately 50 mg of leaf using a modified Qiagen RNeasy Mini kit protocol. The leaf tissue was ground with a mortar and pestle to a fine powder in liquid nitrogen. The Treatments in the lysis step of protocol included RLT (Lysis buffer for lysing cells

Table 1. Geographic and climatic information of the studied populations

Direction	Population	Latitude (N)	Longitude (E)	m.a.s.l. *	Mean annual temperature (°C)	Mean annual precipitation (mm)
West	Shafarod	37° 28' 31.9"	48° 48' 41.2"	1045	11.16	1054
Lowland	Kheyroud lowland	36° 36' 9.6"	51° 34' 7.3"	429	16	1414
Upland	Kheyroud upland	36° 32' 30.7"	51° 38' 39.7"	1136	8.55	1150
East	Shastkalateh	36° 44' 13.9"	54° 24' 14.2"	831	15.4	610

Note: * Meter above sea level.

and tissues), RLC and RLC+ PVP 40000 to a final concentration of 2%. B-mercaptoethanol was added into lysis buffers with the ratio of 10 µL β-ME per 1 ml of buffer and thoroughly mixed. PVP-40 (polyvinylpyrollidone, average molecular weight 40,000) was added in the extraction buffer (RLC buffer) to bind to the phenolic compounds which were then eliminated by ethanol precipitation (Malnoy et al. 2001). Extraction buffer was incubated at 56°C for 2 min. Lysis buffer was added, and the slurry was homogenized using a vortex. The remaining steps were performed according to the manufacturer's protocol. In subsequent preparations, each RNeasy Spin Column was incubated for 5 min at room temperature (15–25°C) after addition of Buffer RW1 and before centrifuging and without the incubation for testing DNA omitting. The concentration and purity of the extracted RNA were analysed by means of a Picodrop spectrophotometer. The integrity of total RNA was determined by running samples on a 1% agarose gel. The gel was stained with ethidium bromide (Sambrook et al. 1989) and visualized using a UV – transilluminator. Also, PCR was performed with 18S rRNA primers (Olbrich et al. 2008) to test for absence of DNA contamination in RNA extractions. PCR was performed in a final volume of 20 µl, containing 50–100 ng of template, 1x reaction buffer, 0.5 mM of each dNTP, 2 nM $MgCl_2$ and 10 pm of each primer and 1 U Taq polymerase. The PCR conditions were as follows: 1 cycle at 50°C for 1 min, 1 cycle at 95°C for 15 min, 35 cycles at 95°C for 15 s, and 60°C for 1 min. Finally, the samples with 260/280 ratio from 1.9 to 2.1 and 260/230 ratio from 2.0 to 2.5 were chosen.

RT-PCR

Reverse transcription and genomic DNA elimination were performed according to the QuantiTect Reverse Transcription kit protocol (Qiagen). RT-PCR amplification was performed with primers specific to the 18S rRNA (Olbrich et al. 2008), as a reference gene. Primers for specific genes were designed using the OLIGO Primer Analysis Software (Molecular Biology Insights 2011) and Vector NTI software package (Life Technologies 2012) (Table 2). To ensure the absence of genomic DNA contamination, primers were designed from 2 exons. Quantitative PCR analysis was performed with a 7300 Real-Time System (Applied Biosystems) thermocycler and a SYBR *Premix Ex Taq* (Tli RNase H Plus) (Takara). The 1.0 µL diluted-cDNA (20 ng total RNA), 10.0 µL SYBR Green Master Mix, 10 pmol of a pair of forward and reverse primers, 0.4 ROX and 6.6 µl of ddH_2O were mixed for the amplification reaction. The PCR program comprised the following steps: pre-incubation for 1 min at 95°C, 40 cycles of amplification for 10 s at 95°C, 31 s at annealing temperature according to Table 2, and for 31 s at 72°C. The data were collected in the last phase (extension phase). To ensure the absence of dimer and non-specific products in amplifications, melting curve analysis was done. Real-time PCR experiments were performed with three identical technical replications.

Data analyses

Relative gene expression was calculated referring to Livak and Schmittgen (2001). Normalized data were used to study differential gene expression in different samples. The relative gene expression comparisons among the four studied populations and two water shortage levels were performed by two-factor factorial analysis of variance in a completely randomized design using the IBM SPSS Statistics software package, version 21.0 (IBM 2012). Also, to find a correlation between genes, the Pearson correlation was performed. Principal component analysis (PCA) was performed using the PAST software package, version 2.17c (Hammer 2001, Hammer et al. 2001). The data were analysed as correlation matrix based on Euclidean distances.

Gene fragment isolation and sequence analysis

Fragments of cDNA were amplified by PCR using primers designed for target genes (specific primers). PCR products were cut from the agarose gel and the fragments purified by using a PCR product extraction kit and Sanger sequenced. Nucleotide sequences were compared with sequences available in the NCBI database using the online application BLAST, and homologous sequenc-

Table 2. Primer sequences and corresponding annealing temperatures for the selected candidate genes

Primer sequence	Annealing temperature	Identification number
F: GTCCAGTGCAGACTATC R: CGTGTCAAAGTGTTAGC	58	FR774766 (Aldehyde dehydrogenase)
F: CATGCTTCATCGCTCTG R: TCTCCTTCACCTTCTCC	58	FR772355 (Dehydrin)
F: CCCTCACTTTCCCGATATTG R: GGCCATTTAACGTCCCTAC	63	FR775803 (Early response to dehydrin)
F: TACCGGATGGACATGATG R: TGAAGCCTAGCAAAAGCC	60	FR796392 (Isocitrate dehydrogenase)
F: TCGGAACCATGAAGCAC R: CTTAACGGCAACAACCC	58	FR774767 (Ascorbate peroxidase)
F: ACTACTTCAACACCACCC R: AGCTCACGCAATTCCATC	60	FR796395 (Transcription factor zinc finger protein)
F: CTCAGGAAAAGGCAAGC R: CTAGCAGTATTAGCAGC	58	AJ130888 (ABA-inducible protein(LEA1))

Table 3. The comparison of mean of leaf morphologic traits in four studied areas: leaf length, leaf width, leaf perimeter, leaf index, leaf shape factor, leaf area, petiole length, distance between the lower point of the lamina and the maximum width point on the axis, the angle between the first sub and primary vein, maximum width index, petiole index, Radius Coefficient and the number of veins were measured

Traits	Shafarod	Kheyroud lowland	Kheyroud upland	Shastkalateh
LL	8.38 ± 0.06 b*	8.87 ± 0.05 a	7.52 ± 0.03 c	8.21 ± 0.09 b
LW	5.12 ± 0.08 a	4.62 ± 0.02 b	4.24 ± 0.07 c	3.78 ± 0.09 d
LP	20.40 ± 0.05 a	20.70 ± 0.10 a	18.04 ± 0.16 c	18.60 ± 0.24 b
LI	171.00 ± 4.01 d	198.67 ± 1.62 b	184.67 ± 2.23 c	227.00 ± 3.87 a
SF	0.84 ± 0.01 a	0.75 ± 0.01 c	0.78 ± 0.01 b	0.70 ± 0.01 d
LA	27.81 ± 0.48 a	25.72 ± 0.33 b	20.24 ± 0.42 c	19.53 ± 0.73 c
PL	0.47 ± 0.01 a	0.40 ± 0.02 b	0.24 ± 0.01 d	0.33 ± 0.01 c
LMW	4.16 ± 0.07 ab	4.34 ± 0.09 a	3.38 ± 0.03 c	3.99 ± 0.13 b
NV	12 -	12 -	12 -	12 -
AN	35.58 ± 1.07 c	52.11 ± 1.16 a	45.04 ± 1.33 b	47.24 ± 1.13 b
MWI	49.66 ± 0.83 a	49.00 ± 1.17 a	44.96 ± 0.27 b	48.28 ± 1.19 a
PI	5.57 ± 0.04 a	4.53 ± 0.20 b	3.19 ± 0.12 d	4.04 ± 0.10 c
RC	0.91 ± 0.01 a	0.87 ± 0.01 c	0.88 ± 0.01 b	0.83 ± 0.01 d

Note: * – the same characters mean no significant difference; LL – leaf length, LW – leaf width, LP – leaf perimeter, LI – leaf index (LI = LL / LW × 100), SF – leaf shape factor, LA – leaf area, PL – petiole length, LMW – distance between the lower point of the lamina and the maximum width point on the axis, AN – the angle between the first sub and primary vein, MWI – maximum width index (MWI = LMW / LL × 100), PL – petiole index (PL = PL / LL × 100), RC – Radius Coefficient (RC = radius area / radius based on the perimeter) and NV – the number of veins were measured.

es found with the highest percentage similarity were selected. Multiple sequence alignment was performed and edited using MEGA 5.1 with the Clustal Omega method (Tamura et al. 2011). A phylogenetic tree was constructed using the UPGMA method with bootstrapping analysis in MEGA5, and the confidence levels of monophyletic groups were estimated using bootstrap analyses of 1,000 replicates.

Results

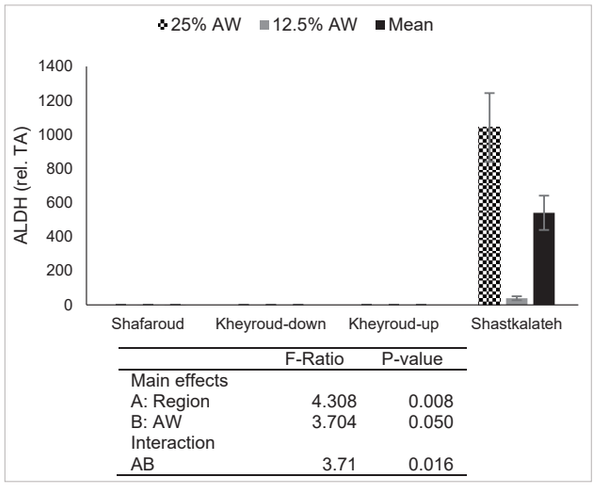
The phenotypic flexibility is a commonly occurring phenomenon in leaf properties in response to environmental factors. The results of the analysis of variance of various leaf traits among four populations showed a significant difference ($\rho = 0.01$) (Table 3). The leaves of individuals from the Shafarod population, which is located in the very humid region, have a greater lamina width, lamina perimeter, and lamina area compared to individuals from the Shastklateh population, which originate from semi-humid region; and the leaves of individuals from the Kheyroud population found at a lower elevation compared to the upland Kheyroud population have a greater lamina length, lamina width, lamina perimeter and lamina area (Table 3).

The results of the evaluation of three classes of the leaf damage at the end of water shortage treatment (25% of available water) showed that Shastklateh seedlings had 70% low damage, 20% high damage and 10% no damage; Kheyroud upland seedlings had 50% low damage and 50% high damage, Shafarod and Kheyroud lowland seedlings similarly had 75% high damage and 25% low damage, and the leaves of all seedlings have wrinkled and malformed by increasing the stress level up to 12.5% of the available water (Figure 2).

The expression level of the aldehyde dehydrogenase gene was higher in the Shastklateh population from dryer region than that of the moister regions, while no significant difference was recorded between the elevation gradients (Figure 3). BLAST results and phylogeny relationship of the *Fagus orientalis* aldehyde dehydrogenase gene (KP989711 sequence) showed that this sequence evolutionally is very similar to the *Fagus sylvatica* gene (95% similarity) and then with genes from 7 other tree species (72–88% similarity) (Figure 4).

Investigations into the expression of ascorbate peroxidase gene, which encodes an enzyme in the decomposition of hydrogen peroxide, showed it was the highest in progenies from the Kheyroud upland and Shastklateh than in those of other regions (Figure 5).

Figure 2. Leaf damage classification (left to right): not damaged, slightly damaged, strongly damaged



	F-Ratio	P-value
Main effects		
A: Region	4.308	0.008
B: AW	3.704	0.050
Interaction		
AB	3.71	0.016

Figure 3. Relative transcript abundance (rel. TA) of ALDH in beech leaves (*F. orientalis*) from the moist to dry regions (Scale bars indicate means ± SE)

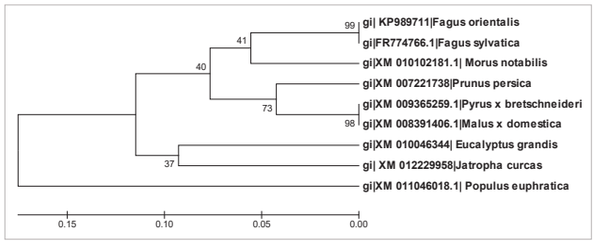
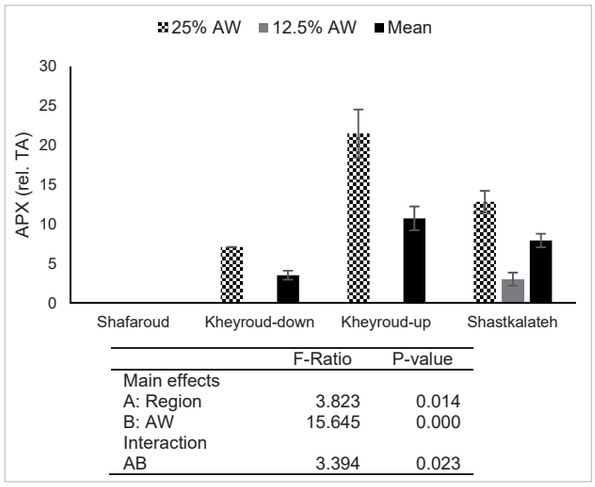


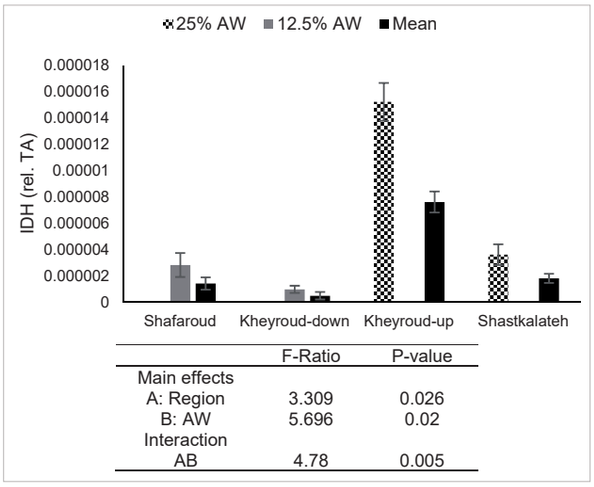
Figure 4. Phylogenetic tree of aldehyde dehydrogenase gene of *Fagus orientalis*



	F-Ratio	P-value
Main effects		
A: Region	3.823	0.014
B: AW	15.645	0.000
Interaction		
AB	3.394	0.023

Figure 5. Relative transcript abundance (rel. TA) of APX in beech leaves (*F. orientalis*) from the moist to dry regions (Scale bars indicate means ± SE)

The expression level of isocitrate dehydrogenase gene was higher in genotypes from the Kheyroud upland and Shastklateh than in those from the other regions (Figure 6). Furthermore, BLAST results and phylogenic relationships of *Fagus orientalis* isocitrate dehydrogenase gene (KP989714 sequence) showed that this sequence was evo-



	F-Ratio	P-value
Main effects		
A: Region	3.309	0.026
B: AW	5.696	0.02
Interaction		
AB	4.78	0.005

Figure 6. Relative transcript abundance (rel. TA) of IDH in beech leaves (*F. orientalis*) from the moist to dry regions (Scale bars indicate means ± SE)

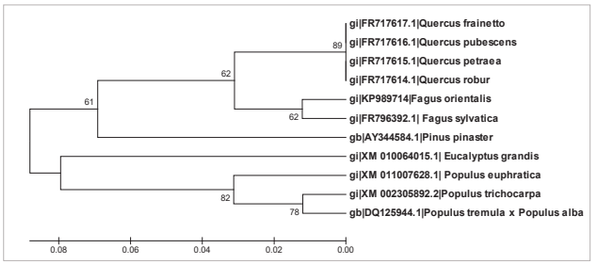


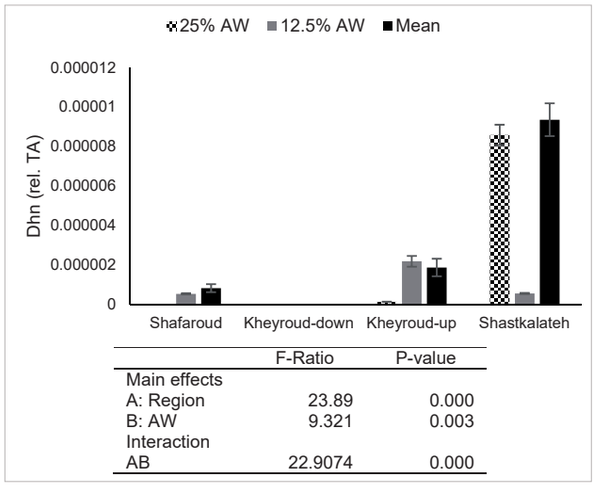
Figure 7. Phylogenetic tree of isocitrate dehydrogenase gene of *Fagus orientalis*

lutionally like the *Fagus sylvatica* gene (95%) and genes from several species of oak and five other tree species (80–90%) (Figure 7).

Also, the results indicated there was a higher level of Dehydrin (DHN) expression as the response gene to water shortage in genotypes from the Kheyroud upland and Shastklateh than genotypes in other regions (Figure 8).

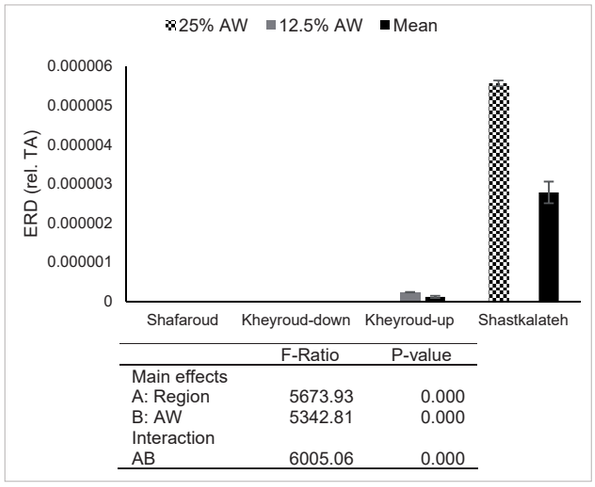
The gene expression level of rapid response to dehydration (ERD) was higher in genotypes from the Kheyroud upland and Shastklateh than other regions and with increasing stress intensity in the treatment of 12.5% available water, the gene expression level is probably reduced due to interruption of the natural cellular interactions (Figure 9). BLAST results and phylogenic relationships of ERD gene sequence of the *Fagus orientalis* (KP989713) showed this sequence in evolutionary terms is similar only to *Fagus sylvatica* species (98%).

The results indicated that LEA1 gene expression level in response to water shortage stress was higher in the Shastkalateh genotypes from the dryer region than the other three from moister regions. However, no difference was observed between the two populations of the Kheyroud upland and the Kheyroud lowland located at different elevations above sea level (Figure 10). With increasing inten-



	F-Ratio	P-value
Main effects		
A: Region	23.89	0.000
B: AW	9.321	0.003
Interaction		
AB	22.9074	0.000

Figure 8. Relative transcript abundance (rel. TA) of DHN in beech leaves (*F. orientalis*) from the moist to dry regions (Scale bars indicate means ± SE)

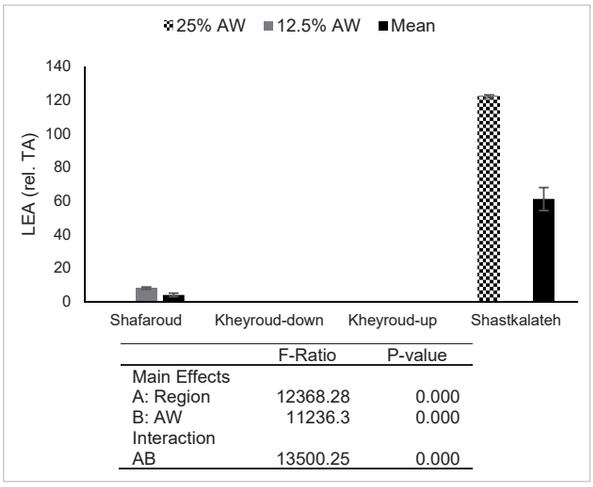


	F-Ratio	P-value
Main effects		
A: Region	5673.93	0.000
B: AW	5342.81	0.000
Interaction		
AB	6005.06	0.000

Figure 9. Relative transcript abundance (rel. TA) of ERD in beech leaves (*F. orientalis*) from the moist to dry regions (Scale bars indicate means ± SE)

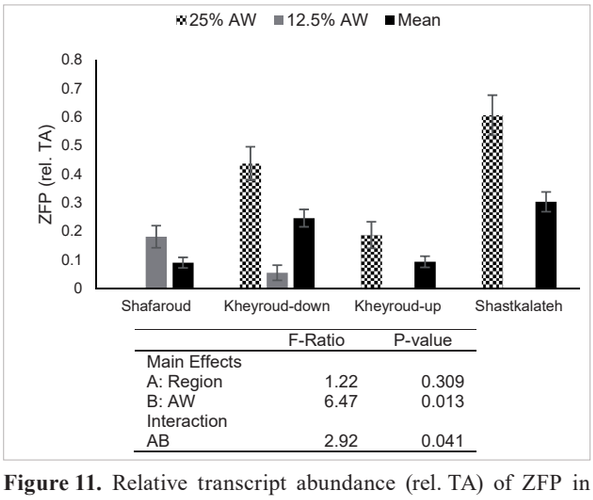
sity of water shortage stress in the treatment of 12.5% of available water, seedlings weakened, and the gene expression level dropped. BLAST results and phylogenic relationships of LEA gene (KP989710) of the *Fagus orientalis* showed that this evolutionary and functional sequence has a similarity only to *Fagus sylvatica* (96%).

Also, Zinc Finger gene (ZFP gene) expression, which is one of the important transcription factors in response to drought stress, was increased in the treatment of 25% of available water in the Shastkalateh population compared to other ones. The gene expression level was reduced with increasing stress intensity and senescence (Figure 11). BLAST results and phylogenic relationships of zinc finger gene (ZFP gene) of the *Fagus orientalis* (KP989709 sequence) showed that this sequence is evolutionally like the *Fagus sylvatica* gene (96% similarity) and then with genes from 7 other tree species (72–83%**?**) (Figure 12).



	F-Ratio	P-value
Main Effects		
A: Region	12368.28	0.000
B: AW	11236.3	0.000
Interaction		
AB	13500.25	0.000

Figure 10. Relative transcript abundance (rel. TA) of LEA in beech leaves (*F. orientalis*) from the moist to dry regions (Scale bars indicate means ± SE)



	F-Ratio	P-value
Main Effects		
A: Region	1.22	0.309
B: AW	6.47	0.013
Interaction		
AB	2.92	0.041

Figure 11. Relative transcript abundance (rel. TA) of ZFP in beech leaves (*F. orientalis*) from the moist to dry regions (Scale bars indicate means ± SE)

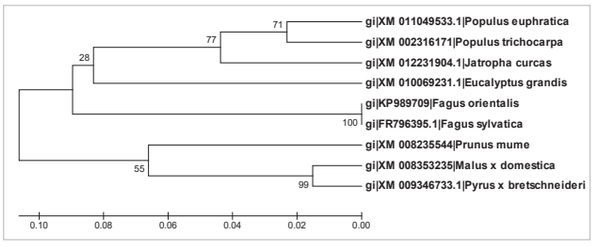


Figure 12. Phylogenetic tree of Zinc Finger gene (ZFP gene) of *Fagus orientalis*

Correlation analyses conducted for the transcript levels of the genes revealed significant relationships for most of the genes studied (Table 4). Exceptions were IDH expression levels, which were not correlated with the expression of any of the other genes.

To determine the main factors responsible for the differences in regions and the response to water short-

Table 4. Pearson correlation between the expressions of the studied genes

Gene	ALDH	ERD	APX	LEA	Dhn	IDH	ZFP
ALDH	1	0.999 **	0.346 **	0.595 **	0.945 **	0.108	0.765 **
ERD		1	0.342 **	0.594 **	0.951 **	0.107	0.764 **
APX			1	0.36 **	0.301 *	0.044	0.286 *
LEA				1	0.942 **	0.118	0.778 **
Dhn					1	0.099	0.712 **
IDH						1	0.310 **
ZFP							1

Note: * significant at 5% error, ** significant at 1% error.

Table 5. The results of sequencing and recording nucleotide sequences

Registered accession numbers for *F. orientalis* in NCBI	DNA fragment length (bp)	Sequencing (5'-3')
KP989709	147	AGACGGGGACCGCTTCGTCCAGACGAGTGGCAAATGTGG-GAAAACAAACTTGACATGCA CTCCACCTGCCTTGCAACTCCCTGGTAG-TCGGCTAAAGACTGCCTTGAGTGCTCGAGAT TTTGAATTGGA-GATGGAATTGCGTGAGCT
KP989710	105	CCCGGGTGGGCCGGGCATGCTGCGTCAGTCTGCCAAGGATACATGCC-TACAAGGCTGG TAAGCTAGTGCCGAGCTAAGGCACTAGGGGCTGCTAA-TACTGCTAGA
KP989711	103	ATAAATAAGAGTACGAGTGAACGAAGAGCAAATTCCTCACAC-CTATGGGCTTGCTGCTGG GGTGTTTACACATAACATAGACAC-TGCTAACACTTTGACACGA
KP989712	135	AAAAAGCTTAGTTCGACCTATGGAGATATGTACGACGTT-GGCTTCCTGAACTTACCAGGCT ACCAACTGAATGGATACAC-CACCCATGGAATGCACCAGAATCTGTACTCCATGCTGCTGG AATT-GAGCTGGGGA
KP989713	115	GACGGTAACTTCTGTACGACTCGACGCCTTCTTCGACGAATACTACGTT-GATCAACATGAA GAAGAAGAGAGAAACAATTTCAAGGATTTGGTTCCAG-TAGGGACGTTAAATGGC
KP989714	84	TTTCAACTTTACTGGGTGCTGGAGGTGTAGCATTGTCCATCGTACAA-TACTGATGAGTCCA TTCGGGCTTTTGCTAGGCTTCAA

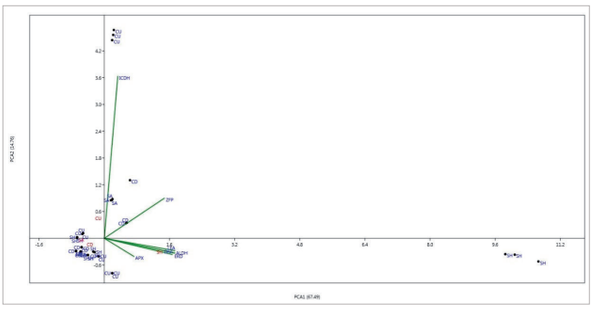


Figure 13. Principal component analysis of all of the studied genes

age, PCAs were conducted for gene expression in different regions and two levels of treatment (Figure 13). PC1 was the main component representing 67.49% of the variation and ALDH, ERD, LEA, DHN and ZFP were the main positive loadings of PC1 with high correlation with *r* values ranging from 0.84 to 0.98. PC2 was the second component representing 14.76% of the variation and IDH was the most significant loading with $r = 0.9$.

The analysed fragments of the 7 genes were amplified and sequenced using the designed primers, and 6 fragments had acceptable sequencing results and were deposited in the NCBI database (Table 5).

Discussion

Plants display various phenotypes in response to environmental changes. The phenotypic flexibility that occurs widely in leaf properties is known as a response to environmental effects, so that thick leaves are considered as a structural adaptation for resistance to wilt in warm, dry and sunny environments. Moreover, large leaves will be more vulnerable to water shortage than smaller ones. The results of leaf morphology study in the west-east gradient and elevation gradient with increasing drought index have shown significant changes in adaptation to the environment in the way that the leaves became smaller and stretched. Also, more morphological changes were observed in the west-east gradient than in elevation one because of increasing drought index. The results of study done by Pyakurel and Wang (2014) on the leaf morphological changes and pore characteristics of birch populations (*Betula papyrifera* Marsh) and their relationship with the geographical and climatic origin of those populations showed that a significant difference was found between the leaf characteristics measured and average annual rainfall and drought slope. Some previous investigations using beech provenances or ecotypes from different geographic regions have already identified phenotypical differences that suggested the presence of adaptive traits (Peuke et al. 2002, Czajkowski and Bolte 2006).

The gene expression involves the process of converting the gene information into a functional product such as the protein and is one of the most important genetic topics that helps produce a specific phenotype from a genotype. Adjusting the gene expression allows the cell to control its structure and function, and this is the basis of morphological differences, and adaptation of living organisms to new conditions.

Different transcripts of plant aldehyde dehydrogenase have been reported in response to environmental stress suggesting the expression of these genes under conditions of water shortage stress and high salinity and their potential role in improving and increasing the osmotic tolerance of the plant (Barclay and McKersie 1994, Kotchoni and Bartles 2003, Sunkar et al. 2003, Bray 2004, Kirch et al. 2004, Kirch et al. 2005, Kotchoni et al. 2006, Rodrigues et al. 2006, Hung et al. 2008). In line with the results of the above-mentioned researches, it can be said that genotypes of Shastklateh as the driest site, with increased expression of aldehyde dehydrogenase gene compared to genotypes of other populations, increased the tolerance to water shortage, and low leaf damage also confirmed this. However, seedlings with increasing water shortage stress intensity in the treatment of 12.5% of available water were senescent and the gene expression was reduced (Figure 3). The research carried out under extreme environmental stress such as drought indicated many usual cellular interactions are damaged and sometimes the damage is irreversible. Under these conditions, the activity of a gene is not necessarily increased unlike the presence of an external stimulus and/or internal signal molecules (Larkindale et al. 2005). The level of aldehyde dehydrogenase expression in the results obtained by Carsjens et al. (2014), in contrast to their initial hypothesis, were constitutively lower and not higher in progenies from dry than from mesic sites, which is probably due to the presence of other different mechanisms for preventing the damage of water shortage stress in *Fagus sylvatica*, which is not consistent with the results of the present study. Also, due to the high similarity of the sequence of this gene with *Fagus sylvatica* it is probable that this gene has a similar function in the production of aldehyde dehydrogenase protein and resistance to water shortage.

Ascorbate peroxide using ascorbate as a specific electron donor reduces H_2O_2 and converts it into water. The expression of ascorbate peroxidase encoding genes is indicated under various environmental stress such as drought and salinity, high light, high and low temperatures, pathogen attack, H_2O_2 and abscisic acid (Teixeira et al. 2004, Bonifacio et al. 2011). Many studies especially on agronomic and herbaceous model species indicate that increased expression of ascorbate peroxidase, superoxide dismutase and aldehyde dehydrogenase enzymes increase protection against drought (Reddy et al. 2004, D'Arcy-Lameta et al. 2006, Kotchoni et al. 2006, Foyer and Noctor 2009, Kar 2011). The results of this research confirmed that the genotypes of the Kheyroud upland and Shastklateh with increased expression of ascorbate peroxidase gene compared to Shafarod and Keyrod had a better resistance system to water shortage and less damaged leaves. The studied seedlings with increasing water shortage stress intensity in the treatment of 12.5% of available water were senescent, and the gene expression level was reduced that in line with other studies, the gene activity was not necessarily increased with increasing stress versus the external stimulus and/or internal signal molecule (Figure 5).

The expression level of isocitrate dehydrogenase gene, which encodes the isocitrate dehydrogenase enzyme and indirectly plays a role in eliminating and preventing the formation of various oxygen species, was higher in genotypes from the Kheyroud upland and Shastklateh than in those from other regions. The expression level of this gene was reduced with increasing stress intensity. Isocitate dehydrogenase depending on nicotine amide dinucleotide phosphate is one of the key elements in supporting the stress response mechanisms (Corpas and Barroso 2014). Several studies have shown that plants exposed to oxidative stress, salinity, and trace metals show higher isocitrate dehydrogenase activity (Galvez et al. 2005, Valderrama et al. 2006, Daniel et al. 2007). Also, due to the high similarity of the sequence of this gene with *Fagus sylvatica* stands as a reliable conclusion that the gene plays a role in the production of the isocitrate dehydrogenase enzyme which is one of the key elements of support mechanisms in response to stress.

Dehydrin proteins belong to LEAII family and accumulate in the plant tissues in response to the loss of cellular water (Tompa and Kovacs 2010). The transcription of LEA gene and the gene protein accumulation have been observed in green tissues of many plants under stress (Colmenero-Flores et al. 1997, Kavar et al. 2008, Caruso et al. 2002, Blodner et al. 2007). In line with the results of the research, it can be concluded that Shastklateh seedlings with increased Dhn gene expression in comparison with other populations, increase tolerance to water shortage, but the seedlings with increasing stress intensity (12.5% of available water) were senescent and the gene expression level was reduced. The research shows that in severe environmental stress, such as drought, many usual cellular interactions are damaged and sometimes the damage is irreversible. Also, rapid induction of DHN expression was observed after osmotic stress in a hybrid poplar (*Populus euramericana*) cluster (Caruso et al. 2002). Similar increases have been also observed in the transcription or LEA family protein levels in other forest trees, such as *Picea* (Blodner et al. 2007).

The gene expression level of early response to dehydration (ERD) was higher in the seedlings genotypes from the Kheyroud upland and Shastklateh than in those from other regions. ERD genes are known as the genes that are quickly activated during drought stress. The expression of these genes increases the response to the drought, low

temperature, high salinity, and abscisic acid, resulting in quick and effective mechanism responses and preventing the damage to the chloroplast membrane (Steponkus et al. 1998, Taji et al. 1999).

LEA1 gene encodes late embryogenesis abundant protein. This protein is a group of hydrophilic proteins that accumulate under the influence of water stress, low temperature, salinity and acetic acid in the growing organs. The results indicated that LEA1 gene expression level was higher in the seedlings from Shastkalateh than in those from other three regions under discussion in response to water shortage stress. LEA protein plays a role in protecting and helping stabilize macromolecules, including chlorophyll and protein complex, as well as preventing membranes and other proteins from drying because of the water molecules it contains (Maitra and Cuhman 1994). Different LEA proteins are known to accumulate against the environmental stress in the plant, thus they should interact with the mechanisms of resistance through accumulation in the tissue in the early stages of the emergence of stress.

Also, zinc-finger protein gene expression (ZFP gene), which is one of the important transcription factors in response to the drought stress, was higher in the treatment of 25% of available water in in the seedlings from Shastkalateh population compared to other populations. Several studies show that several transcription factors play a role in response to the drought stress, including WRKY, zinc-finger, MYC, MYB, NAC, Bzip, AP2/ERP, etc. (Soren et al. 2012). Zinc-finger transcription factor is one of the transcription factors that is strongly induced by H_2O_2 and contributes to oxidative stress (Petrov and Breusegem 2012). Also, due to the high similarity of the sequence of this gene with *Fagus sylvatica*, it seems that this gene has the same role in the production of zinc finger transcription factor, which plays an important role in response to drought.

The existence of a significant and positive correlation between most of the studied genes could mean that the ability of *Fagus orientalis* to resist water shortage uses a wide range of the genes inducing water shortage stress that directly or indirectly causes stress resistance. Aldehyde dehydrogenase, isocitrate dehydrogenase and ascorbate peroxidase genes with their enzymatic role in the removal of toxic aldehyde and hydrogen peroxide and Dehydrin (DHN), early responsive to dehydration (ERD), late embryogenesis abundant (LEA) genes with the production of hydrophilic proteins and zinc-finger protein gene (ZFP gene) with the production of a transcription factor can lead to resistance to water shortage.

Conclusion

The present study shows the adaptability of oriental beech to drought. The pressure of natural selection in past evolutionary periods in the eastern regions has led to more adaptation in the Shastkalateh population compared to the populations of the western regions. Also, based on the significant difference in the most leaf traits and expression of drought response genes in the west-east gradient populations, it was found that the effect of longitude is higher than elevation in beech adaptation. Therefore, the western and lowland populations are more likely to be exposed to the risk of damages due to drought and heat effects because of climate change. Hence, risk management of such populations should be considered. By effective risk management, effects of climate change such as displacement of vegetative boundaries, prolongation of growth period and increased vulnerability to late cold and development of pests due to physiological weakness of the trees in water shortage conditions can be minimized. The main management approach seems to be the development of genetic resources by reducing human damages, removal of aqueous competitor species to reduce competition for water resources, identification of more resistant trees for natural and artificial regeneration and reducing the intensity and increasing periods of thinning to help natural selection.

In general, according to the results of this study, Oriental beech populations can be evaluated for resistance to drought stress by analysis of the most effective genes responding to water shortage stress and leaf traits in the Hyrcanian forests.

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