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Expression profiles of 11 candidate genes involved in drought tolerance of pedunculate oak (*Quercus robur* L.). Possibilities for genetic monitoring of the species.

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Abstract

Pedunculate oak (*Quercus robur* L.) is one of the most significant broadleaved tree species in Europe. However, various abiotic and biotic agents have influenced pedunculate oak forests, among which drought stress has been frequently described as the main driver of this species forests decline. In this study we assessed relative expression profile of 11 candidate genes involved in many different metabolic pathways and potentially responsible for oak drought tolerance. The obtained results succeed in partially tackling drought tolerance mechanisms of targeted natural pedunculated oak population. This gene pool may represent a base for adaptation and therefore genetic diversity should be conserved. In this paper we described different expression responses of four pedunculate oak ecological groups, characterized by different physiological status (senescent vs vital) and flowering period (early (var. *praecox*) vs late (var. *tardissima*)). The most significant differences in relative gene expression levels are shown between the flowering period (*tardissima* (8 genes upregulated) vs *praecox* (3 genes upregulated)), more than a physiological status (senescent vs vital). Only three genes *wrky53*, *rd22* and *sag21* showed upregulated expression pattern in senescent physiological groups, indicating their possible role in the coping mechanisms of oak in stressed environment. Results showed interesting connections of relative gene expression values of

identified drought-tolerance related genes with flowering period and provide further recommendations for adequate conservation and monitoring of this important oak gene pool in its southeast refugium.

Keywords: *adaptation, drought, gene expression, pedunculate oak, qPCR*

Introduction

General projections for climate changes are that they will intensify in the near future, with emphasis on the intensity and frequency of climate extremes (e.g. drought, temperature, precipitation) (IPCC, 2021). It is thus important to understand how plants respond to climate, especially forest tree species which are sessile organisms directly influenced by various abiotic and biotic stressors. A suite of functional traits affect water relationships, resource acquisition and other aspects of plant function (Manzoni, 2014) mediates vegetation responses to environmental conditions. For instance, drought tolerance and water use efficiency of trees can be significantly affected by genetic and environmental background and their interaction (Voelker et al., 2014; Pilipović et al., 2020), but still little is known about

molecular mechanisms involved in these traits (Trudić et al., 2012).

Temperate oaks represent one of the major components of the European broadleaved forest resources with high economic and ecological importance, contributing greatly to the social and economic well-being of the populations (Varela, 1995; Ducouso, A., and Bordacs, S., 2003). The regeneration of pedunculate oak in Serbia is based on the shelterwood cutting system in large areas, where the understory layer and trees of the previous stand are removed in several phases in short regeneration periods (2-3 years), through the preparatory, regeneration and final cuts. However, even though present management models give good results, changes and improvements are needed considering the presence of multiple biotic (pest and diseases) and abiotic factors (e.g. drought stress) that have negative influence on the survival and development of pedunculate oak forests (Rađević et al., 2020). Marker assisted genetic analyses of pedunculated oak in Serbia, which is the south-eastern refugium, showed presence of conserved heterozygosity (Trudić et al., 2021), meaning that forest management keeps good selection practices in this area allowing sustainability of the gene pool.

Abiotic stresses are major causes of loss of forest production and natural vegetation because they cause many morphological, physiological, biochemical and molecular changes that affect plant growth and productivity (Trudić et al., 2012). Arend et al. (2013) recorded significantly higher metabolic sensitivity to drought stress in *Quercus robur*, compared to two other common oak species in Western Balkans (*Q. petraea* and *Q. cerris*), which makes pedunculate oak an interesting study area for further adaptability research. Indeed, other studies evidenced large intraspecific variability of intrinsic water-use efficiency (WUEi) in *Q. robur* (Ponton et al., 2002), as well as high contribution of certain leaf stomatal traits in driving WUEi diversity on a species level (Roussel et al., 2009; Stojnić et al., 2019a), further indicating that this parameter might be efficiently used in breeding programs as a suitable indicator of *Q. robur* drought sensitivity. Knowledge about, and „management“ of abiotic stresses are especially important for long-term growth and survival of woody plant species worldwide. Adaptation of plants to stress from the environment is mediated by a series of highly coordinated and complex signaling pathways. Stress-sensing mechanisms regulate different cellular and molecular events in plants and the expression of various stress-tolerant genes (Trudić et al., 2013). Pilipović et al. (2020) conducted a study of the influence of simulated drought on the pedunculate oak seedlings, measuring net photosynthesis, stomatal conductance and chlorophyll content. Results showed that there was a significant genetic effect in relation to the seedlings response to induced stress and recovery, indicating the possibility to use physiological parameters in the selection of stress-tolerant oak progenies and provenances.

One of the ways plants react to abiotic stress is a change in the expression of the corresponding genes (Trudić et al., 2012). Products from the activities of these genes can be divided into two groups: 1) regulators of gene expression and signaling pathways (e.g. protein kinases, phosphatases, transcription

factors, etc.), and 2) functional molecules that are directly involved in tolerance to stress, such as various antioxidants, chaperones, osmotic protectants, etc. (Trudić et al., 2013).

Large collections of oak expressed sequence tags (ESTs) have been generated from various tissues and developmental stages, including 130,000 Sanger sequences and approximately 2.5 million reads available from public databases (Ueno et al., 2010). This catalog constitutes a useful resource for detecting candidate genes controlling traits of interest for the development of new genetic markers for genetic approaches (linkage mapping and QTL detection, association mapping) or for dissection of genetic architecture of adaptive traits (Alberto et al., 2010; Casasoli et al., 2006; Derory et al., 2010; Durand. et al., 2010; Leroy et al., 2020). Additionally, the reference genome of pedunculate oak is now available and anchored to a genetic map, which makes it easier to identify genes that matter the most for adaptation of this species (Plomion et al., 2018).

Taking into consideration the importance of pedunculate oak gene pool for local, national and regional biodiversity and mitigation of climate change, profound importance is addressed to genetic diversity research within *ex situ* and *in situ* conservation efforts (Stojnić et al., 2019b). In addition, breeding of more resilient provenances represents one of the efficient ways for future adaptation that will enable plants to optimize their life processes in prevailing environmental conditions on the evolutionary scale (Beikircher and Mayr, 2009). Therefore, the aim of this study was to give the first insight on the molecular mechanism explaining drought tolerance in targeted *Q. robur* population and generate recommendations important for conservation efforts, using relative expression profiles of 11 genes involved in drought stress tolerance in plants.

Material and Methods

Sampling of specimens

In order to properly evaluate individuals for further gene expression analysis, we first selected them according to phenophase (early (var. *praecox*) vs late (var. *tardissima*)) and physiological groups (vital or senescent), and then we assessed morphometric traits for all of them. The main criterion for determining the affinity of trees to one of the physiological groups (vital or senescent) was the degree of canopy damage, which was assessed by Dubravac et al., (2011): trees with canopy damage above 25 % were considered significantly damaged trees, while trees with canopy damage up to 25 % were considered undamaged or vital trees. Damage caused by pathogens was used as an additional criterion in tree selection. Canopy damage was assessed in the same year as sampling.

Leaf samples from 42 individuals (21 individuals of *Quercus robur* var. *praecox* (11 vital + 10 senescent) and 21 individuals of *Q. robur* var. *tardissima* (11 vital + 10 senescent)) were collected from seed orchard forest located in the Northern part of Serbia (N 45° 00' 12.66" E 019° 08' 50.4") during springtime in April and May 2013 (Figure 1). The altitude of the management

unit was ranging from 81 to 83 m above sea level (a.s.l.). The geological substrate was defined as alluvial sand sediments with mostly gley soils ranging from riparian black soil and black meadow soil to brown forest soil. Hydrological conditions were characterized by the absence of flooding and soil moisture was strongly dependent on fluctuations of groundwater table levels. The investigated area represents Pannonian environmental zone PAN1 (Metzger et al., 2005). The climate records were obtained from nearby weather station „Sremska Mitrovica“ (N 45°01“; E 19°33“; 82 m a.s.l) for the period 2011-2013 (Republic Hydrometeorological Service of Serbia 2011, 2012 and 2013) (Supplementary material 1).

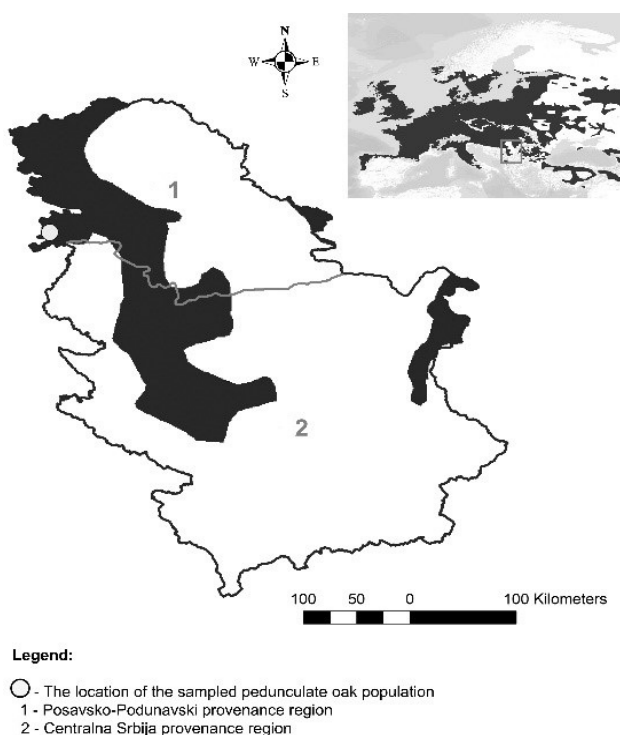


Figure 1

The map of two officially registered regions of pedunculate oak provenances in Serbia regulated by national decision on establishing the regions of pedunculate oak provenances in Serbia: 91/2008-16. Shaded areas represent the distribution range of *Q. robur* in Europe (upper smaller picture) and Serbia (large picture) according to Ducouso, A., and Bordacs, S. (2003). The small white dot in upper left part of the map is marking the location of pedunculate oak gene pool covered in this study.

Morphometric analyses

For all individual trees, diameter at breast height (DBH [cm]), absolute height of trees (H [m]) and height to the crown (HC [m]) were performed. DBH was measured with classic caliper (Haglöf, Sweden) whereas H and HC were measured using "Vertex III" (Haglöf Sweden AB) apparatus. All statistical analyses for morphometric parameters were performed in Statistica version 13 (TIBCO Software Inc. 2017). One-way analysis of variance (ANOVA) was conducted to determine the statistical

significance of the four oak ecological groups to (1) DBH, (2) H and (3) HC. All statistical analyses were performed at $p \leq 0.05$ level of significance.

DNA extraction, PCR and qPCR

Forty-two fully developed leaf samples were harvested and sent on dry ice via DHL transportation service 24 hours before the start of RNA extraction to BIOGECO laboratories in Bordeaux, Cestas, France. To minimize the variability between individuals as much as possible, we performed extraction independently for 10 senescent and 11 vital individuals separately and then generated three biological replications equimolarly for each condition and variety. Total RNA was isolated from samples following the procedure described in Le Provost et al. (2007). The quantity and quality of the RNA were assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) after removal of residual genomic DNA with DNase I enzyme and purification step with RNeasy RNA clean up protocol[®] by QIAGEN. Process of reverse transcription and construction of cDNA was done by iScript cDNA Synthesis Kit[®] from Bio-Rad Company according to the manufacturer's instructions. Two housekeeping genes (*18s* with 103 % efficiency and *ef β* with 99 % efficiency) were used to normalize the data and the main criterion for their selection was their stability across biological samples.

Prior to gene primers design in Primer 3[®] software (Rozen and Skaletsky, 2000) a search of gene sequences for *Arabidopsis thaliana* was done in TAIR database (The Arabidopsis Information Resource, 2015). After that, Blast analysis was performed against oak EST database in order to identify and select orthologues based on their e-value (Table 1 and Table 2).

We checked the specificity of the amplification product using conventional agarose gel electrophoresis and afterwards we performed the quantification. Conventional PCR programs for multibanding quality control check were as follows: initial denaturation at 94°C for 5 min, 35 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, and final extension at 72°C for 7 min.

Master mix solution for qPCR for a total of 12 samples was prepared as a mixture of forward and reverse primers (3.6 μ l of each, see Table 2.), mQH₂O (88.8 μ l) and Tp IQSyberGreen (120 μ l). qPCR reaction was run for 18 μ l of Master mix solution with 2 μ l of cDNA sample, following standardized program for this type of biological samples and reaction: initial denaturation at 95°C for 3 min, 39 cycles at 95°C for 15 sec, 95°C for 45 sec; plate read, melting curve from 65 to 95°C, read every 0.1°C, hold 1 sec on Chromo4 Continuous Fluorescence Detector Gradient Cyclor DNA engine™ (Chromo4™ System - Bio-Rad). A threshold for DNA-based fluorescence detection was set slightly above background and the threshold cycle (C_t) was measured as described in Le Provost et al., 2016.

First, geNorm (<https://genorm.cmgg.be/>) was used to select the most stable housekeeping genes before qPCR analyses. Mean C_t and their standard deviations were calculated for individual samples and then pooled into four ecological groups: early senescent oak (hereinafter: PEOS), early vital oak (hereinafter: PEOV), late senescent oak (hereinafter: PLOS) and

Table 1

Candidate genes used for qPCR expression profile determination. Each gene was checked for multibanding pattern through conventional PCR reaction and agarose gel electrophoresis.

No.	Annotation	Gene	Primer pairs	GenBank accession number
1	Drought induced gene 21	<i>atdi21</i>	F: ATTTCGAAGCTCGACTGCAT R: AGAAGATTGTGGGGCCTCTT	CR627776
2	AWPM-19-like membrane family protein	<i>awmp</i>	F: GTTGCTTCATGCTGGGATCT R: CATAAGGGTGGTGTCTCTGT	XM_031067968.1
3	Gibberellin-regulated GASA/GAST/Snakin family protein	<i>gasa</i>	F: GATGTATGCCCTGCTACG R: ATGGCTGCATGTGTTGGTA	XM_031099810.1
4	Basic 7S globulin 2	<i>globulin</i>	F: GCCACTGCCATTCCAATAT R: AGGAGCGGCATATTCATTG	XM_024060619.1
5	Glutathione-s-transferase tau 19	<i>gst</i>	F: CGGCGTAGAGGAACGATTAG R: TGTCCTTTAGCTTGCAAGA	XM_031105862.1
6	Late embryogenesis abundant protein Lea5	<i>lea</i>	F: AGAAGATTGTGGGGCCTCTT R: ATGCAGTCGAGCTTCGAAAT	XM_031068332.1
7	Dehydration-responsive protein RD22 precursor	<i>rd22</i>	F: GCCCAGACAACATGATCCTC R: CAGAATGGAACCAAGCAT	FP041436
8	Senescence associate gene 21	<i>sag21</i>	F: CTCGTCGACACGATTCTCAG R: GGTGAAGAAGCAAGGTGAGG	XM_031092942.1
9	Gene for transcription factor wrky involved in defense, wounding and senescence 53	<i>wrky53</i>	F: GGGAGCGATGAATTTGAGAA R: CCCTCACTTGGTCTGTCCAT	FN727229
10	Dehydrin Xero 2	<i>xero2</i>	F: GCTTTGACACTTGCTTTTGC R: AAACCAGCAGAAGAAGGGGT	AM711636.1
11	Xyloglucan endotransglucosylase/hydrolase 9	<i>xet2</i>	F: TGATCCATGCCTGTTGGTA R: AAGTGGTCTAAACCGCCCT	XM_031074399.1

Table 2

Genes used in this study and their role in plant metabolism. Plant organism(s) – organism in which the gene was firstly identified and reported.

Gene	Role	Plant organism(s)	Reference
<i>atdi21</i>	Response to abscisic acid stimulus, response to water deprivation, response to stress.	<i>Arabidopsis thaliana</i>	Aghdasi et al., 2012
<i>awmp</i>	Response to temperature stress.	<i>Gossypium hirsutum</i>	Hsing et al., 1995
<i>gasa</i>	Response to gibberellin stimulus.	<i>Arabidopsis thaliana</i>	Thierry-Mieg et al., 2006.
<i>globulin</i>	Seed storage protein. Response to stress, hormone receptor-like activity, protein kinase activity. Binds leginsulin.	<i>Glycine hispida</i>	Hirano, 2021
<i>gst</i>	Response to drought stress, oxidative stress, and high doses of auxin and cytokinin.	<i>Arabidopsis thaliana</i>	Wagner et al., 2002
<i>lea</i>	Response to abiotic stress (dehydration, salinity, high and low temperature).	<i>Gossypium hirsutum</i>	Dure et al., 1981
<i>rd22</i>	Response to salt stress, response to abscisic acid stimulus, response to desiccation.	<i>Arabidopsis thaliana</i>	Yamaguchi-Shinozaki and Shinozaki, 1993
<i>sag21</i>	Response to cold, response to oxidative stress, response to stress	<i>Arabidopsis thaliana</i>	Miller et al., 1999
<i>wrky53</i>	Defense response to bacterium, incompatible interaction, regulation of transcription.	<i>Arabidopsis thaliana</i>	Murray et al., 2007
<i>xero2</i>	Cold acclimation, response to abscisic acid stimulus, response to cold, response to water deprivation, response to stress.	<i>Arabidopsis thaliana</i>	Chung and Parish, 2008

late vital oak (hereinafter: PLOV). All calculations were done in Gene Expression Analysis for iCycler iQ v1.10™ from Bio-Rad using calculations derived from the algorithms outlined by Vandesompele et al., (2002). Bar charts were created using matplotlib v3.4.1 package (Caswell et al., 2021) for Python programming language for pooled samples as differences in gene expression between these groups is the main aim of this study. The Python script for the bar chart creation is available on request.

Results

Morphometric analyses

The results ANOVA for DBH, H and HC between genotypes with different ecological status are shown in Table 3. No statistically significant differences between ecological types in terms of investigated phenotypic traits were found.

Table 3

Variance results of diameter at breast height (DBH[cm]), absolute height of trees (H[m]) and height to the crown (HC[m]) between genotypes with different ecological status with $p \leq 0.05$ level of significance. df – degrees of freedom; SS – sum of squares; MS – mean square; F – F statistic.

Effect	df	DBH				H				HC			
		SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Ecological type	3	218.1	72.7	0.841	0.479	86.9	28.9	2.632	0.064	24.6	8.2	1.096	0.363
Error	37	3197.7	86.4			407.2	11.0			277.4	7.5		
Total	40	3415.9				494.1				302.1			

Relative gene expression

Results of descriptive statistics (means and standard deviations) of threshold cycle (Ct) and relative gene expression (RGE) of all genes for each oak category are presented in Table 4 and depicted on Figure 2. Maximum relative gene expression was 7,27 for PLOV for *gasa* gene, whereas minimum values for relative gene expression were 1,00 for PEOS for *awpm* and *xero2* genes, PEOV for *atdi21*, *gasa*, *lea*, *rd22*, *sag21* and *wrky53*, and PLOS for *globulin*, *gst* and *xet* genes.

Discussion

During 2011–2012, a very dry and long summer with higher temperature, strong insolation, lesser amount of available water in the soil and atmosphere, by multifactorial drought, made the environment quite challenging for survival of pedunculate oak forests in the observed area (Stojanović et al., 2015). In this study, we wanted to obtain first insight to differential relative gene expression response to these complex environmental changes.

Certain ecological groups of analyzed oak showed high and very high values of standard deviation. This was expected since samples were taken from individuals grown in nature where multifactorial ecological influences on gene expression is present, including the variation of different flowering periods. Higher gene expression was observed within phenological groups (late vs early ones) rather than between physiological conditions (8 genes vs 3 genes). *Wrky53* and *rd22* clearly showed upregulation pattern within groups identified as senescent, indicating initiative expression during the physiological processes of abiotic stresses, especially cumulative drought. Similarly, Spies et al. (2012) found that genes with protective function such as *lea* and *rci2b* had increased transcript levels in *Quercus robur* under drought stress. Porth et al. (2005) characterized 33 osmotic-stress-induced transcripts from tissue cultures of sympatric species to pedunculate oak, *Q. petraea*.

They also found immediate increase of expression of the *badh* in response to osmotic stress in *Q. petraea* species, but the progression of gene expression for *badh*, *ltp* and *ox2og* differed strikingly between *Q. robur* and *Q. petraea*. Additionally, expression of the *badh*, which catalyzed the synthesis of the osmoprotective molecule glycine betaine, was down-regulated in *Q. robur*, whereas it was up-regulated in *Q. petraea* (Porth et al., 2005). Expression of the *ltp*, responsible for formation and reinforcement of plant surface layers, was rather constant in *Q. robur* compared with *Q. petraea* (Porth et al., 2005). The high expression of *ltp* in *Q. petraea*, which is adapted to dry sites, likely reflects species specificity in expression level (Porth et al., 2005). Unfortunately, they did not monitor these gene expression patterns with bud phenology nor any other correlation with it.

Following RGE patterns of *wrky53* and *rd22*, *sag21* gene showed similar expression pattern, although the highest value of RGE was shown in late flowering vital group. This mechanism is not surprising since buds are made of dehydrated tissue in order to avoid ice formation from winter frost (Čehulić et al., 2019). Interesting RGE pattern was also found for *globulin* gene, where significant increase of the expression is shown in

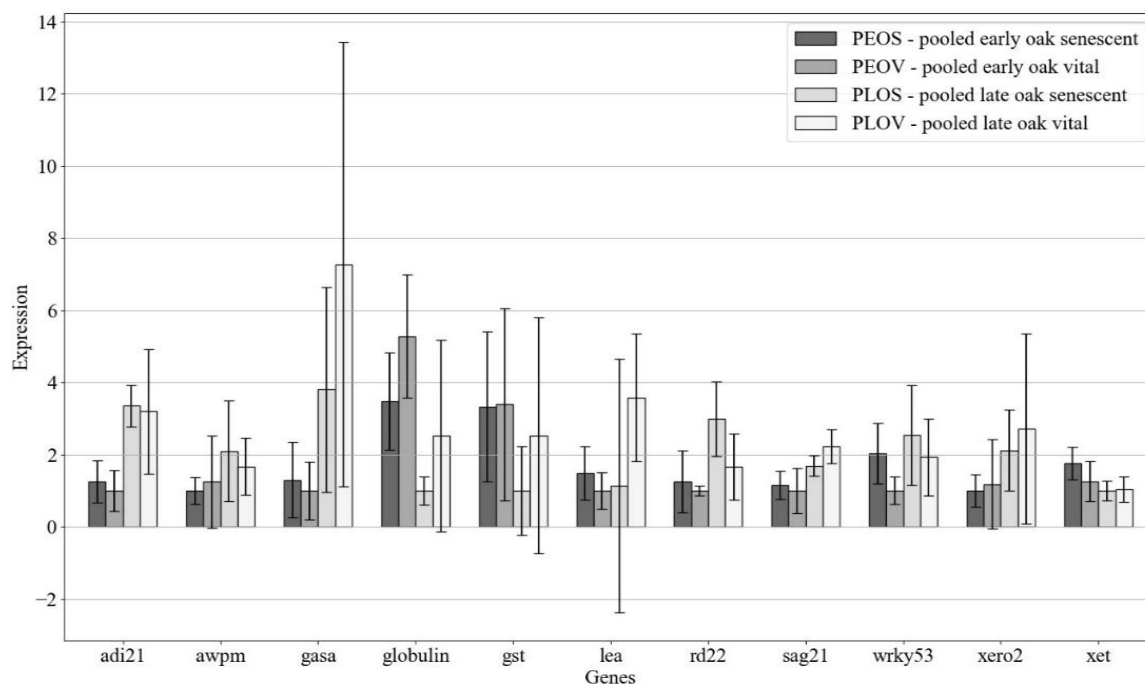


Figure 2

A bar chart of gene expression of 11 genes for four oak categories plotted on X axis and relative gene expression plotted on Y-axis. The data obtained by quantitative RT-PCR were normalized to the expression of the reference genes *efβ* and *18s*. Error bars indicate standard deviation obtained from three biological replicates. Values are the mean of three technical replicates of independent cDNA synthesis from the same extraction. Pooled representation of all individuals belonging to each ecological group was used to get the insight of gene expression response. See Appendix 2 of supplementary material for bar charts of each individual gene relative expression profile per oak ecological group.

early flowering senescent group whereas this expression significantly decreases in late flowering senescent group. It might be that this gene is active during early flowering phases supporting plants metabolism to cope with the both physiological processes of bud burst and senescence caused by external abiotic stressors. *Adi21* gene showed similar high RGE pattern in late flowering senescent group, probably having similar role as *globulin* but in later flowering periods. These two genes highlight different molecular strategies implemented in the different oak ecological groups to cope with drought stress conditions.

Kotrade et al., (2019) used quantification data from a comparative transcriptome analysis for the selection of candidate reference genes for three oak species (*Q. robur*, *Q. ilex* and *Q. pubescens*) for stress transcriptomic analysis and identified novel reference genes for each species (*fhy3* for *Q. robur* and *at1g54610* for all tree species) that are more stably expressed than classical reference genes. It is therefore expected that by testing a larger set of reference genes, more genes that are highly stably expressed across all oak species could be identified and later used in stress-related studies.

Conclusions

A more accurate picture on gene expression profiling and metabolic reactions on drought stress should be facilitated with the comparison of our results with the ones where samples were grown in controlled conditions, like seedlings and tissue culture in greenhouse environment. This way, particular gene function can be tackled, and biology of species accurately understood when it comes to the abiotic and biotic stress influence on gene expression pattern. Our goal is to develop such approach in the coming years to target among the chosen candidate genes those that matter for adaptation to drought in oaks.

Although described limitations within the facilitated study do not allow gaining full conclusion on the adaptation of oak to long-term drought exposure in open environment, it is clear that oak trees are capable of adjusting their metabolism for some time, at the cost of a phenotype quality and overall vitality. Also, we registered significant correlation of bud burst phenology with majority of observed genes. Previous studies through literature review showed that *Q. robur* also applies different strategies of drought stress protection depending on the duration and intensity of the stress. It is needed to involve

Table 4

Mean C_t and relative gene expression (RGE) \pm standard deviation (SD) of 11 genes for four analyzed types of oak. PLOS: pooled late oak senescent, PLOV: pooled late oak vital, PEOS: pooled early oak senescent, PEOV: pooled early oak vital. Bolded RGE \pm SD indicate very high values of standard deviations particularly comparing to the value of RGE. Bolded and underlined values are the ones where SD values are higher than the RGE values.

Gene	Oak ecological group	C_t	RGE \pm SD
<i>adi21</i>	PEOS	21.37 \pm 0.689	1.25 \pm 0.578
	PEOV	22.21 \pm 0.860	1.00 \pm 0.559
	PLOS	20.20 \pm 0.164	3.35 \pm 0.577
	PLOV	20.43 \pm 0.831	3.20\pm1.729
<i>awpm</i>	PEOS	23.61 \pm 0.493	1.00 \pm 0.371
	PEOV	23.75 \pm 1.461	1.25\pm1.278
	PLOS	22.89 \pm 0.928	2.10\pm1.391
	PLOV	23.36 \pm 0.667	1.67\pm0.792
<i>gasa</i>	PEOS	23.80 \pm 1.229	1.30\pm1.043
	PEOV	24.71 \pm 1.240	1.00\pm0.800
	PLOS	22.50 \pm 1.146	3.80\pm2.845
	PLOV	21.65 \pm 1.313	7.27\pm6.164
<i>globulin</i>	PEOS	24.83 \pm 0.675	3.48 \pm 1.352
	PEOV	24.65 \pm 0.580	5.28 \pm 1.704
	PLOS	27.60 \pm 0.675	1.00 \pm 0.387
	PLOV	26.06 \pm 1.958	2.52\pm2.661
<i>gst</i>	PEOS	22.04 \pm 1.008	3.33\pm2.084
	PEOV	22.53 \pm 1.290	3.39\pm2.661
	PLOS	24.42 \pm 2.007	1.00\pm1.220
	PLOV	23.05 \pm 2.131	2.53\pm3.271
<i>lea</i>	PEOS	20.76 \pm 0.744	1.48\pm0.737
	PEOV	21.87 \pm 0.789	1.00 \pm 0.514
	PLOS	21.54 \pm 4.783	1.14\pm3.515
	PLOV	19.92 \pm 0.759	3.58 \pm 1.772
<i>rd22</i>	PEOS	19.06 \pm 1.011	1.26\pm0.856
	PEOV	19.89 \pm 0.145	1.00 \pm 0.129
	PLOS	18.11 \pm 0.479	2.99 \pm 1.028
	PLOV	19.15 \pm 0.830	1.66\pm0.921
<i>sag21</i>	PEOS	20.58 \pm 0.413	1.15 \pm 0.391
	PEOV	21.19 \pm 0.823	1.00\pm0.625
	PLOS	20.38 \pm 0.135	1.69 \pm 0.287
	PLOV	20.15 \pm 0.254	2.23 \pm 0.470
<i>wrky53</i>	PEOS	23.97 \pm 0.595	2.03 \pm 0.840
	PEOV	25.53 \pm 0.569	1.00 \pm 0.384
	PLOS	23.99 \pm 0.795	2.54\pm1.375
	PLOV	24.56 \pm 0.823	1.93\pm1.060
<i>xero2</i>	PEOS	21.75 \pm 0.612	1.00 \pm 0.446
	PEOV	21.97 \pm 1.515	1.18\pm1.243
	PLOS	21.01 \pm 0.739	2.12 \pm 1.122
	PLOV	20.79 \pm 1.388	2.72\pm2.625
<i>xet</i>	PEOS	21.31 \pm 0.307	1.76 \pm 0.445
	PEOV	22.24 \pm 0.626	1.26 \pm 0.558
	PLOS	22.46 \pm 0.358	1.00 \pm 0.283
	PLOV	22.55 \pm 0.470	1.04 \pm 0.352

larger groups of genes and sample size in order to get precise information on gene expression profiles with multifactorial abiotic stress influence. In case of natural populations, which are under influence of many climatic factors sometimes it is difficult or impossible to measure, continuity and longevity of gene expression monitoring as a part of regular forest genetic monitoring practice is needed.

We recommend establishing continuous, long-term monitoring of ecophysiological parameters (photosynthesis, transpiration, stomatal conductance and other plant water status related parameters) which can be correlated with the expression of particular genes or family of genes directly involved in those and related processes *in planta*. In terms of further usage of these results in forest genetic monitoring practices of pedunculate oak and related broadleaved species, further principle coordinate analyses of relative gene expression values and other available parameters such as expected and observational heterozygosity, phenotypic measurements, physiological and biochemical measurements are needed. This would make possible to determine which set of parameters is the most reliable in genetic monitoring practice of the species.

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