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Expression profiles of 12 drought responsive genes in two European (deciduous) oak species during a two-year drought experiment with consecutive drought periods

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ABSTRACT

Based on an earlier comparative transcriptomics study (Madritsch et al., 2019, in revision) 12 representative genes were selected that were differentially expressed in response to drought stress in two major European oak species, pedunculate oak (*Quercus robur*) and pubescent oak (*Q. pubescens*). RT-qPCR technique was used to validate the quantification data from the transcriptomics study and to generate detailed gene expression profiles over the course of a two-year drought experiment with repeated long-term drought events comprising eight sampling dates. The 12 genes were selected based on quantification data and on their functional annotation and belong to different pathways and phytohormone signaling networks. *PP2C27* (Protein phosphatase 2C 27), *RD22* (Response to desiccation 22) and *STP13* (Sugar transport protein 13) are key molecules of the abscisic acid (ABA) signaling pathway that plays a major role in the modulation of the molecular drought stress response. *AOS1* (Allene oxide synthase 1) is the first enzyme in the biosynthesis of jasmonates (JAs), *ERF4* (Ethylen responsive transcription factor 4) is part of the ethylene-activated pathway and *CDR1* (Constitutive disease resistance 1) is part of the salicylic acid (SA) dependent pathway. Two genes related to the phenylpropanoid pathway were included, *PAL* (Phenylalanine ammoniase-lyase) catalyzes the first step of the pathway and *UGT73C6* (UDP-glycosyltransferase 73C6) plays a role in the biosynthesis of quercetins and other flavonoids, these contribute to tolerance against oxidative stress. *NRX2* (Nucleoredoxin 2) also plays a role in protection against oxidative stress. Also, two genes that are related to photosynthesis, *BCA2* (Beta carbonic anhydrase 2) and *GPT2* (Glucose-6-phosphate/phosphate translocator 2) and the nutrient transporter *BOR2* (Boron transporter 2) were included. Most genes revealed clearly different expression profiles over the course of the experiment, indicating an adjustment process during the long-term repeated drought periods. Despite being part of the same signaling network, the ABA-related genes showed divers expression profiles, reflecting the complexity of the drought response and the modulation of the response over the course of the experiment in the two *Quercus* species. The expression profiles for *AOS1*, *ERF4* and *PAL* indicate that they were upregulated by JA pathway in *Q. pubescens* independently from drought stress treatment, contradicting an involvement of JA pathway to the drought stress response. *BCA2*, *NRX2*, *PP2C27* showed a contrary regulation in the two species that might be linked to their different drought tolerance while *BOR2* and *UGT73C6* were shown to be drought responsive for the first time.

1. Introduction

Drought events are a major threat for agricultural and natural vegetation (Ciais et al., 2005; Lesk et al., 2016; He et al., 2018). Therefore, the molecular response of plants to drought stress was studied intensely for the last few decades, mostly in dicot model organisms such as *Arabidopsis thaliana* and tobacco or in monocot crop species such as maize, rice and barley (Shinozaki and Yamaguchi-Shinozaki, 2007; Fahad et al., 2017).

Generally, drought is perceived through the root system of the plant and leads to an increased production of the phytohormone abscisic acid (ABA) and the activation of ABA-dependent and -independent gene networks that alter the expression of drought responsive genes (Shinozaki and Yamaguchi-Shinozaki, 2007). Under persisting drought

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stress, ABA levels start to decrease gradually and the long-term drought response is fine-tuned in orchestration with other phytohormones such as ethylene, brassinosteroids, salicylic acid (SA) and jasmonic acid (JA) (Yang et al., 2014). The drought responsive genes can be classified as regulatory or functional genes. The regulatory genes comprise transcription factors, protein kinases, phosphatases and key enzymes of phytohormone biosynthesis, while the functional genes play a role in detoxification, osmoprotection and the protection of macromolecules or transportation (Shinozaki and Yamaguchi-Shinozaki, 2007).

While molecular drought stress response is well investigated in some herbaceous species, only a few studies have assessed the molecular response in forest trees (Harfouche et al., 2014). Since the molecular drought response depends on species-specific anatomical and physiological traits, as well as drought survival strategies (Kooyers, 2015), it also varies between genotypes of the same species (Guo et al., 2009). Therefore, the knowledge acquired so far on the molecular drought response cannot be simply transferred to forest tree species without verification. Furthermore, it must be considered that the stress response also depends on drought intensity, duration and desiccation rate (Bray, 1997; Clauw et al., 2015). Most drought studies in herbaceous species have assessed the short-term response to rapid desiccation which is a rather unnatural experimental design regarding European forest tree species because drought events that cause damage in European forests typically have a slow onset and are long-lasting.

Since climate change is expected to exaggerate drought periods in Europe, challenging natural forest structures (Hanewinkel et al., 2013; Bennett et al., 2015), it is important to elucidate drought resistance mechanisms in forest tree species. Clarifying the molecular drought stress response of tree species may improve our understanding of the impact of climate change on European forests and may lead to the identification of molecular drought resistance markers. Since assisted migration and breeding projects are approaches to preserve forests and their economic and ecological value through climate change (McLachlan et al., 2007; Vitt et al., 2010), these markers could facilitate the targeted selection of genotypes and could help reduce the risks and costs of assisted migration and breeding projects (Collard and Mackill, 2008).

In Europe, 11 of the 36 most abundant forest tree species belong to the genus *Quercus* (Köble and Seufert, 2000), covering approximately 24% of potential forestry areas (Hanewinkel et al., 2013). However, up to now, only three studies assessing the transcriptomic response have been conducted for the deciduous European oak species *Quercus robur*, *Q. pubescens* and *Q. petraea* (Porth et al., 2005; Spieß et al., 2012; Torre et al., 2014). These studies revealed, inter alia, differences in drought induced gene regulation between *Q. petraea* and *Q. robur*, provided functional markers associated with mechanisms of drought avoidance in *Q. pubescens* (Torre et al., 2014) and generated gene expression profiles of drought responsive genes by using the cDNA microarray technique in *Q. robur* (Spieß et al., 2012). In only one of these studies were long-term drought events repeatedly applied under controlled conditions (Spieß et al., 2012).

1.1. Objective

This current study is part of a primary project that aims to identify genes that are linked to drought stress resistance in European oaks in order to facilitate the development of marker gene assays for breeding and assisted migration projects. A lysimeter drought stress experiment was conducted in 2014 and 2015 with three major European oak species, the deciduous pedunculate oak (*Q. robur*), the pubescent oak (*Q. pubescens*) and the evergreen holm oak (*Q. ilex*), which have an ascending drought tolerance, in that order. Physiological data from the experiment was published recently (Früchtenicht et al., 2018a, 2018b). Also, a cross-species comparative transcriptomics study was conducted with leaf samples from a single sampling date in the first year of the experiment (Madritsch et al., 2019, in revision), leading to the identification of 415, 79 and 222 differentially expressed genes (DEGs) during drought stress in *Q. robur*, *Q. pubescens* and *Q. ilex*, respectively.

Based on the transcriptomics study by Madritsch et al. (2019, in revision), 12 representative DEGs between drought stress and control groups with regulatory or functional tasks were selected for the current study. Whereas the transcriptomics study generated a snap-shot of gene expression of one sampling date in the first year of the drought stress experiment, the objective of the current study is to give a detailed impression of gene expression over the whole course of the two-year experiment under natural conditions, i.e. slow desiccation and the application of repeated long-term drought stress in the two deciduous European oak species *Q. pubescens* and *Q. robur*, thus helping to elucidate the molecular drought stress response in the genus *Quercus*.

2. Materials and methods

2.1. Plant material and drought stress treatment

Detailed information on the drought stress experiment and physiological data of the trees has recently been published (Früchtenicht et al., 2018a, 2018b). Nine-year-old oak trees were planted in two basins with controlled ground water level at −1.8 m: Trees were grown for two years to allow for root development. Three plants of *Quercus robur* (provenance: Mitteldeutsches Tief- und Hügelland D-81705), five plants of *Q. pubescens* (provenance: Languedoc F-QPU701) and three plants of *Q. ilex* (provenance: Languedoc F-QIL 702) were grown in each basin. All plants were provided by Darmstädter Forstbaumschule (Darmstadt, Germany). The evergreen species *Q. ilex* was not chosen as a subject in the present study. The basins were filled with silty loam (24.2–24.9% clay; 12.3–16.3% sand; 59.6–63% silt; field capacity 49.3%) and covered with a semi cylindrical foil tunnel. The trees were not treated with fertilizer during the experiment.

In 2014, one basin was selected for drought stress treatment (DS), the other one served as the control (CO). Irrigation of the DS basin was stopped on DOY (day of the year) 170 for 124 days while the CO basin was kept at a constant water table. After rewatering of the DS basin, both basins were kept at the same water level again. In 2015, irrigation of the DS basin was stopped on DOY 107 and rewatering started after 140 days of withholding irrigation.

The level of drought stress in the plants was monitored by measuring predawn water potential (Ψ_{PD}) with a Scholander pressure chamber (SKPM 1400; SKYE Instruments, UK). The results of the monitoring are listed in Table 1. For detailed information see Früchtenicht et al. (2018a).

2.2. Sampling

Leaf samples were taken consistently from the same three trees per treatment and species, except for the control group of *Q. robur* where only two trees were sampled since one individual perished in mid-2014. Per tree, three to five fully developed leaves of upper branches in the southern orientation were sampled. Leaves were immediately shock-frozen in liquid nitrogen and kept at −80 °C until RNA extraction.

Samples were taken on eight sampling dates in 2014 and 2015, four per year. The first sampling date was a control date on DOY168 in 2014 (D1–14) before irrigation stop on DOY170 and, hence, before any significant differences in Ψ_{PD} occurred between the CO and DS groups. In *Q. pubescens*, significant differences between the groups were observed on the third sampling date in 2014 (D3–14) and on the second and third sampling dates in 2015 (D2–15, D3–15). In *Q. robur*, significant differences between the groups were observed from the second to fourth sampling dates in 2014 (D2–14 to D4–14) and the first to third sampling dates in 2015 (D1–15 to D3–15). The drought stressed trees recovered between the two years; when irrigation was stopped on DOY107 in 2015 (IS-15) no significant differences in Ψ_{PD} were detectable in both species. In addition, on the last sampling date in 2015

Table 1
Mean predawn water potential (ΨPD) of the trees on the four sampling dates in 2014 (D1–14 – D4–14), before irrigation stop in 2015 (IS-15) and on the four sampling dates in 2015 (D1–15 – D4–15) in control (CO) and drought stress treatment (DS) groups. Gray indicates the sampling date for the RNA-Seq. Significant differences between the ΨPD of DS and CO groups are marked with asterisks ($p < .05$). DOY: Day of year.

	Sampling date	D1–14	D2–14	D3–14	D4–14	IS-15	D1–15	D2–15	D3–15	D4–15
Q. pubescens	DOY	168	245	287	323	107	160	203	243	271
	ΨPD CO [MPa]	−0.31	−0.37	−0.14	−0.37	−0.16	−0.27	−0.25	−0.37	−0.17
	ΨPD DS [MPa]	−0.34	−0.57	−0.47	−0.31	−0.15	−0.38	−1.33	−2.24	−0.24
	Sign. diff.	–	*	*	–	–	–	*	*	–
Q. robur	*DOY*	168	245	287	323	107	154	182	191	270
	ΨPD CO [MPa]	−0.42	−0.45	−0.39	−0.59	−0.14	−0.34	−0.19	−0.3	−0.29
	ΨPD DS [MPa]	−0.36	−2.18	−1.63	−1.19	−0.23	−0.79	−1.74	−2.27	−0.33
	Sign. diff.	–	*	*	*	–	*	*	*	–

(D4–15), trees of both species recovered and no differences in Ψ_{PD} were observed.

The RNA-Seq was conducted with samples from the sampling date D3–14 whilst the RT-qPCR experiments were conducted with samples from all eight sampling dates. Sampling dates, corresponding DOY and mean predawn water potential of the CO and DS groups are listed in Table 1.

2.3. RNA sequencing and analysis

Detailed information on the RNA-Seq and comparative bioinformatics analysis is given in Madritsch et al. (2019; in revision).

2.4. Selection of target genes

Six genes that were significantly upregulated (false discovery rate < 0.05) in *Q. pubescens* and six genes that were significantly upregulated in *Q. robur* were manually selected from the RNA-Seq data for detailed analyses by RT-qPCR. Only those target genes with an available functional annotation were selected to allow an assignment to different pathways, so avoiding redundant results and to obtain a representative set of genes. Genes that were not expressed in individual samples according to the RNA-Seq (CPM = 0) were not considered for the study to avoid problems in the RT-qPCR analyses caused by missing Cq-values.

AOS1, *BAC2*, *ERF4*, *GPT2*, *PAL* and *STP13* were selected from the *Q. pubescens* data set, while *BOR2*, *CDR1*, *NRX2*, *PP2C27*, *RD22* and *UGT73C6* were selected from the data set of *Q. robur*. An overview of the selected target genes, annotations and corresponding pathways is given in Table 2. Detailed information on the target genes, including *p*-value, false discovery rate (FDR), log fold change (logFC), transcript abundancy (CPM), NCBI accession numbers and transcript sequences are listed in the appendix in supplementary Table 2.

2.4.1. ABA signaling pathway

PP2C enzymes are key elements in plant signal transduction and *PP2C27* (Protein phosphatase 2C 27) was shown to be a key molecule in the ABA-dependent drought stress response (Rodriguez, 1998; Liu et al., 2012). *RD22* (Response to desiccation 22) is responsive to both drought and ABA and is a key enzyme of the modulation of the photosynthetic apparatus in response to drought stress (Yamaguchi-Shinozaki and Shinozaki, 1993; Harshavardhan et al., 2014). *STP13* (Sugar transport protein 13) is a high-affinity hexose transporter that was identified as a key regulator in a drought responsive hierarchical genetic network in poplar species (Jia et al., 2017).

2.4.2. Ethylene, JA and SA signaling pathways

AOS1 (Allene oxide synthase 1) is the first enzyme in the lipoxygenase pathway that leads to the biosynthesis of jasmonates (JAs); these are involved in response to biotic and abiotic stresses, including drought (Creelman and Mullet, 1995; Wasternack and Parthier, 1997). *ERF4* (Ethylene responsive transcription factor 4) is part of the ethylene-activated pathway and is involved in the abiotic stress response in *A. thaliana*, inter alia, by modulating the ethylene and abscisic acid response (Yang et al., 2005; McGrath et al., 2005). *CDR1* (Constitutive diseases resistance 1) is an aspartic protease and a key regulator in the SA dependent defense response to biotic stress (Xia et al., 2004; Prasad et al., 2009).

2.4.3. Phenylpropanoids and detoxification

PAL (Phenylalanine ammoniase-lyase) catalyzes the first step of the phenylpropanoid pathway and is induced by biotic and abiotic stresses (Guo and Wang, 2009). *UGT73C6* (UDP-glycosyltransferase 73C6) plays a role in the biosynthesis of quercetins and other flavonoids which function as antioxidants and enhance oxidative and drought tolerance (Lim et al., 2004; Nakabayashi et al., 2014). *NRX2* (Nucleoredoxin 2) is a thioredoxin reductase, these enzymes form part of the stress defense mechanisms of plants and contribute to stress tolerance against oxidative and drought stress (Cha et al., 2015).

2.4.4. Photosynthesis

The chloroplastic beta carbonic anhydrase *BCA2* (Beta carbonic

Table 2
Gene names, functional annotations and corresponding pathways of the 12 selected target genes. ABA: Abscisic acid; JA: Jasmonic acid; SA: Salicylic acid.

Gene symbol	Functional annotation	Corresponding pathway
AOS1	Allene oxide synthase 1, chloroplastic	JA – Key enzyme of JA biosynthesis
BCA2	Beta carbonic anhydrase 2-like, chloroplastic	Photosynthesis – Regulation of chloroplastic carbon dioxide levels
BOR2	Boron transporter 2	Transporter – Boron balance
CDR1	Aspartic proteinase CDR1-like	SA - Key regulator in the SA dependent defense response to biotic stress
ERF4	Ethylene-responsive transcription factor 4	Ethylene-activated pathway (also induced by ABA, JA)
GPT2	Glucose-6-phosphate/phosphate translocator 2, chloroplastic	Photosynthesis – Photosynthetical acclimation; Stomatal conductance
NRX2	Nucleoredoxin 2	Detoxification – Protection against oxidative stress
PAL	Phenylalanine ammonia-lyase-like	Phenylpropanoids – Key enzyme of phenylpropanoid metabolism
PP2C27	Protein phosphatase 2c 27	ABA – Signal transduction
RD22	BURP domain protein responsive to desiccation 22-like	ABA – Key regulator of ABA signaling pathway
STP13	Sugar transport protein 13-like	ABA – Key regulator of ABA signaling pathway
UGT73C6	UDP-glycosyltransferase 73C6-like	Phenylpropanoids - Biosynthesis of quercetin and other flavonoids; Detoxification

anhydrase 2) probably supplies RuBisCO with carbon dioxide and has been linked to drought stress tolerance in several plant species (Moroney et al. 2001; Sun et al., 2016; Wang et al., 2016). *GPT2* (Glucose-6-phosphate/phosphate translocator 2, chloroplastic) enables the uptake of glucose-6-phosphate into chloroplasts and is essential for the photosynthetic acclimation to changing environments and involved in stomatal conductance by regulating starch synthesis in guard cells (Athanasiou et al., 2009; Prasch et al., 2015).

2.4.5. Nutrient balance

BOR2 (Boron transporter 2) plays a role in the transport of boric acid/borate from the symplast to the apoplast under boron deficiency (Miwa et al., 2013); an interrelation of boron availability and drought stress tolerance has been documented in various species (Hajiboland and Farhanghi, 2011; Abdel-Motagally and El-Zohri, 2018; Naeem et al., 2018).

2.5. RNA extraction, primer design and RT-qPCR

The detailed information according to the MIQE guidelines that specify the minimal information that must be reported on a qPCR experiment (Bustin et al., 2009) is provided in supplementary text File A.

Briefly, RNA extraction was conducted using a modified CTAB protocol for woody plants (Gambino et al., 2008). Primers that function in both species were designed if possible. For this purpose, multiple sequence alignments comprising all homolog transcripts of both species were created for every target gene using MEGA7 software (Kumar et al., 2016) and conserved sequences were identified. Primers were designed using Primer3 (Untergasser et al., 2012) and tested for specificity with Bowtie2 (Langmead and Salzberg, 2012). DNase I, RNase-free (Thermo Fisher Scientific, USA) was used for DNA digestion and Thermo Scientific Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific, USA) was used for cDNA synthesis. The qPCR reactions were performed on a TOptical thermocycler (Analytik Jena, Germany) using the QuantiNova SYBR Green PCR Kit (Qiagen, Netherlands). Raw data was used to determine Cq values with Real-time PCR Miner, an algorithm which is independent of the equipment used and settings (Zhao and Fernald, 2005). Efficiencies were determined by cDNA dilution series (Pfaffl, 2001). PCR products were checked by melting curves and gel electrophoresis. Reference genes used for the normalization of qPCR data were validated for drought stress experiments in *Q. robur* and *Q. pubescens* in a previous study (Kotrade et al., 2019). In accordance with that study, *At1g54610* and *FHY3* were used as reference genes for *Q. robur* while *At1g54610* and *U2AF35B* were used for *Q. pubescens*. Primer sequences, product sizes and reaction efficiencies for target and reference genes are listed in Table 3.

2.6. Validation of RNA-Seq quantification

In order to validate the quantification data from the RNA-Seq, the data were correlated with quantification data from RT-qPCR experiments. Normalized quantification data from RNA-Seq (CPM values) were $\log_2$ transformed. ΔCq values were calculated to normalize data from RT-qPCR ($\Delta Cq = Cq_{\text{target gene}} - Cq_{\text{reference gene}}$). Scatter plots were generated by plotting the $\log_2$ CPM value against the ΔCq value for each target gene in each biological replicate, resulting in 72 data points for *Q. pubescens* and 60 data points for *Q. robur*. Excel (Microsoft, USA) was used to visualize the data and to calculate the coefficient of determination (R^2) of the data sets.

2.7. Gene expression analyses

The determined Cq values were used to calculate the efficiency corrected, normalized relative expression values for each gene on each sampling date with the software Rest 2009 (V2.0.13; Qiagen, Netherlands). This software uses a pair-wise reallocation randomization test to assess statistical significances of the differences between the tested groups (Pfaffl et al., 2002).

The gene expression profiles were visualized using Excel (Microsoft, USA). Cq values were transformed to normalized relative quantities (NRQs) (Hellemans et al., 2008) and the ratios of the NRQs of DS and CO groups were calculated (equivalent to normalized fold expression values) and standard errors were determined (Rieu and Powers, 2009).

3. Results

3.1. Validation of RNA-Seq quantification

ΔCq values from the RT-qPCR experiments were plotted against $\log_2$ transformed CPM values from the RNA-Seq and the coefficient of determination (R^2) was calculated. In both species a clear correlation was found between the quantification methods, $R^2 = 0.792$ in *Q. pubescens* and $R^2 = 0.7446$ in *Q. robur* (Fig. 1).

3.2. Gene expression profiles

qPCR experiments were conducted with samples from all eight sampling dates to generate gene expression profiles for the 12 selected DEGs. Normalized Relative Fold Expression and statistics were computed with REST2009 software. In the following paragraphs, genes with similar expression patterns are presented jointly. All results are listed in detail in supplementary Table 2.

3.2.1. AOS1, ERF4 and PAL

AOS1, ERF4 and *PAL* show similar expression profiles in *Q. pubescens* (Fig. 2a, c, e). An increasing upregulation is visible during the first year (2014) of the drought experiment, with significantly different expression between the CO and DS groups on D3–14 and D4–14, while in the second year (2015) no significant regulation was detectable. In *Q. robur*, a significant downregulation of *PAL* was determined on D3–14, D4–14 and D4–15 (Fig. 2b) while for *AOS1* and *ERF4* no significant differences were found (Fig. 2d, f).

3.2.2. BCA2, GPT2 and STP13

In *Q. pubescens*, for *BCA2*, *GPT2* and *STP13,* an increase of gene expression in the DS group was found on the second sampling date in 2014 (D2–14) and a significant upregulation was detected on the third date (D3–14), but, in contrast to the first group of genes, expression returned to near-control values on the fourth date (Fig. 3a, c, e). In 2015, no significant up- or downregulation was observed. *Q. robur* reacted differently, exhibiting (non-significant) downregulation for BCA2 and (significant) upregulation for GPT2 in both years and for STP13 in the first year (Fig. 3b, d, f).

3.2.3. BOR2, CDR1, PP2C27 and UGT73C6

This third group of genes was characterized by an increased expression in *Q. robur* in the first year, but not in the second. *BOR2* and *CDR1* were found to be significantly upregulated on D3–14 and D4–14 (Fig. 4b, d), while *PP2C27* was found to be significantly upregulated from D2–14 to D4–14 (Fig. 4f) and for *UGT73C6* an upregulation was only found on D3–14 (Fig. 4h). In *Q.* pubescens, for *BOR2* an upregulation was found in both years (Fig. 4a) and for *PP2C27* a downregulation was found on D2–15 (Fig. 4e), whereas, for CDR1 and *UGT73C6* (Fig. 4c, g), no differential expression was detected at all.

3.2.4. NRX2 and RD22

NRX2 and *RD22* showed upregulation in *Q. robur* in both years of the experiment. *NRX2* was significantly upregulated on all sampling dates from D2–14 to D2–15 (Fig. 5b) while *RD22* was found to be significantly upregulated on D3–14 and on the first three dates in 2015 (D1–15 to D3–15; Fig. 5d). On D2–14 and D4–14 no significant differences between the CO and DS groups were detected despite similar

Table 3
List of target and reference genes with corresponding primer sequences, PCR reaction efficiencies (± standard deviation) and product size.

Gene symbol target genes	Species	Sequences (5′–3′; forward/reverse)	Efficiency ± SD	Product size
AOS1	*Q. pubescens*	CCCAAACATGGTCAAGTGGA/ CTCTTCGGCTAACTGGGTGT	93.75 ± 1.43	64 bp
	Q.robur	”	”	”
BCA2	*Q. pubescens*	CTGAATTGTACGAGAAACTTGCTG/ CCAGGATATGAGAGGGACACA	99.88 ± 3.42	72 bp
	Q.robur	GATCTGTCTCTTGCAATCCAGTT/ TTCTCAGTAACAAGGATGATGTGG	97.13 ± 2.03	93 bp
BOR2	*Q. pubescens*	TCTTGTACCAGTGCGACAGT/ GGGGCTTCCTCATATTCTGC	98.34 ± 2.31	90 bp
	Q.robur	”	”	”
CDR1	*Q. pubescens*	GGAACCCACCCAATGCATG/ GGTAACTTGGCCCAGTCCA	94.51 ± 2.43	161 bp
	Q.robur	”	”	”
ERF4	*Q. pubescens*	ACCACGAAACTCTCTGGCA/ TGGCTTGGAACCTTTGACAC	96.51 ± 2.90	72 bp
	Q.robur	AGGTCGATGTGAATAGGCAGT/ AACAACAGCAAGAAACGCATC	93.88 ± 1.89	72 bp
GPT2	*Q. pubescens*	TTGCAGCCCAAAGTGTCTTC/ TTGATGGGTTGGACAGGTGT	93.61 ± 2.06	165 bp
	Q.robur	CCACGCTGCTCTGTTTTCTT/ CACAACCCGAAAGCTTCCTC	98.36 ± 2. 44	160 bp
NRX2	*Q. pubescens*	TCTGCCTCTCATGCTTCTCC/ CCGAGCCTTCCCGTTTACTA	93.48 ± 0.74	74 bp
	Q.robur	”	”	”
PAL	*Q. pubescens*	TGGAGAAGAGGTAGTGAAAGCT/ CTCACCTTCTTTGCACCAGT	98.65 ± 2.76	118 bp
	Q.robur	GCACTCAGAACCTCTCCTCA/ GAGCAACATCGATCAATGGGT	96.37 ± 1.87	150 bp
PP2C27	*Q. pubescens*	TGCCGACTTCCCTTTAGAACT/ TGCATCAGTCTCCATAAATGACC	99.67 ± 1.68	61 bp
	Q.robur	”	”	”
RD22	*Q. pubescens*	CCATGCAACAGAGACCACAC/ TGCCCAGACAACATGATCCT	94.44 ± 1.84	196 bp
	Q.robur	”	”	”
STP13	*Q. pubescens*	CACCAGAAACACCAACATCGT/ TTGAGGCCAAGATCACACCT	99.76 ± 0.96	151 bp
	Q.robur	TTGCTTGGTTGTGGAGTTGG/ TGCCGTAATTGACAAGGTTGG	98.65 ± 1.95	93 bp
UGT73C6	*Q. pubescens*	GAGCCCAGATTCAACAGCAC/ TCACCACAACACATAACGCA	97.12 ± 2.05	65 bp
	Q.robur	”	”	”
Gene symbol reference genes	Species	Sequences (5′–3′; forward/reverse)	Efficiency ± SD	Product size
At1g54610	*Q. pubescens*	GATGATGCTCCTGGGTTTCC/ AGAGTCACGAGCCATTCCAG	100.02% ± 1.55	200 bp
	Q.robur	”	”	”
FHY3	*Q.robur*	TGACCGTTAGCACACAAGAC/ GCCCCTGAATGGTCTGTTGA	98.71% ± 1.51	171 bp
U2AF35B	*Q. pubescens*	GAGCAACATCAGCAGCATGA/ TCCACAGACAATGACAGCCA	98.01% ± 2.04	77 bp

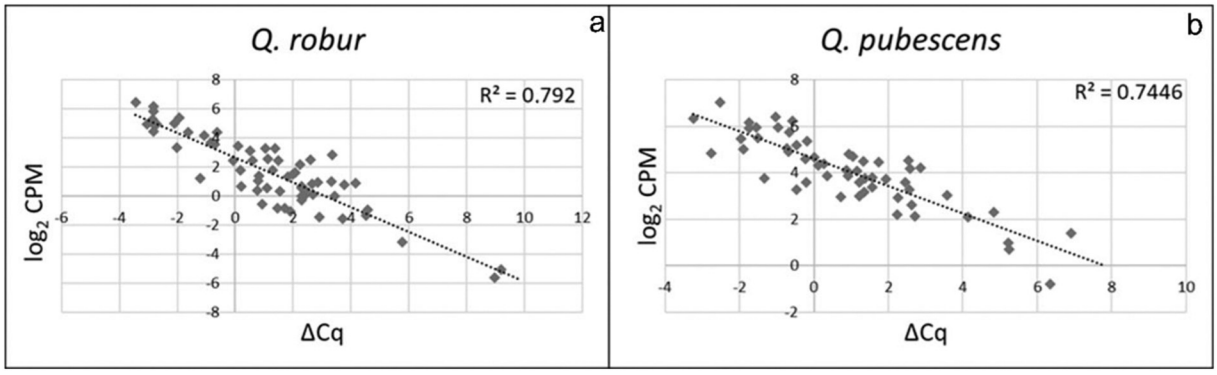

Fig. 1. Validation of RNA-Seq quantification data by RT-qPCR. ΔCq values from RT-qPCR were plotted against $\log_2$ transformed CPM values from RNA-Seq. Each data point represents one quantified target gene in one individual. R^2 = coefficient of determination; a: Q. pubescens; b: Q. robur.

NRQ values as on D3–14 (D2–14: 6.9, D3–14: 6.6, D4–14: 6.2). In *Q. pubescens*, a significant downregulation of *NRX2* was detected on D4–15, while a significant upregulation of *RD22* was detected on D3–15 (Fig. 5a, c).

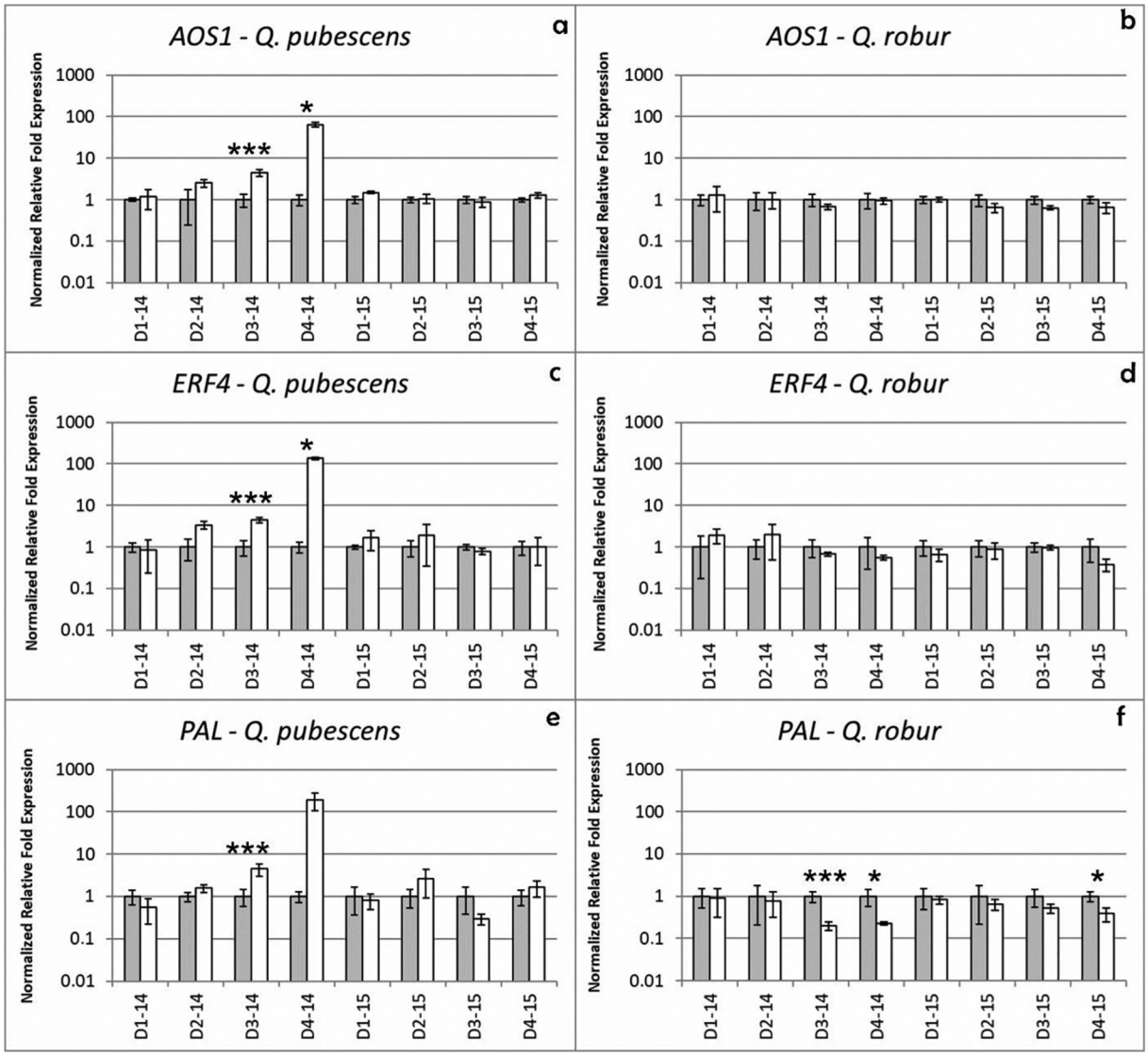

Fig. 2. a-f - Gene expression profiles of the genes AOS1, ERF4 and PAL in Q. pubescens and Q. robur comprising the eight sampling dates D1–14 to D4–15 in 2014 and 2015. Gray bars: CO group; White bars: DS group; Significances are marked with asterisks (* = $p < .05$; *** = $p < .001$), error bars indicate standard error.

4. Discussion

4.1. Validation of RNA-Seq quantification

Since ΔCq values are proportional to the log2 transcript concentrations of samples, a correlation is expected when plotting ΔCq values against $\log_2$ transformed CPM values. The coefficients of determination of $R^2 = 0.79$ and $R^2 = 0.74$ in *Q. pubescens* and *Q. robur*, respectively, showed a clear correlation between the quantification methods and are in accordance with values from other studies using this validation method (e.g. Xiao et al., 2013; Fan et al., 2015). It is noteworthy that the same biological replicates were used for sampling, although different batches of leaves were used for the comparative transcriptomics and RT-qPCR studies. When using the same batch for all applications, an R^2 closer to 1 can be expected.

4.2. Progress of DS

In 2014, the DS *Q. pubescens* experienced only mild stress (ΨPD < -0.5 MPa) while the DS *Q. robur* encountered severe drought stress (ΨPD < -2 MPa) 75 days after the withdrawal of irrigation (Table 1); the latter did not fully recover 48 h after rewatering on the last sampling date in 2014 (D4–14: ΨPD = −1.19 MPa). Before the second drought treatment was applied in 2015 the DS *Q. robur* trees had recovered (IS-15), but on the first sampling date in 2015, which was set 47 days after the irrigation stop, they already showed lowered ΨPD values (D1–15: ΨPD = −0.79 MPa). In 2015, severe drought stress was obtained in both species 136 days after the withdrawal of irrigation (D3–15). DS trees of both species fully recovered by the last sampling date in 2015 (D4–15; 23 and 24 days after rewatering in *Q. robur* and *Q. pubescens*, respectively). For details see Früchtenicht et al. (2018a, 2018b).

Although *Q. pubescens* and *Q. robur* are closely related and belong to the same subgroup in the genus *Quercus* (Denk and Grimm, 2010), the observed slower onset of drought stress in *Q. pubescens* can be explained by its higher drought tolerance due to its better adaptation to more southern European and drier areas compared to *Q. robur* (Früchtenicht et al., 2018a).

4.3. Gene expression

None of the DEGs are expressed similarly in the two subsequent years. For *Q. pubescens* the difference in drought stress intensity between the years (ΨPD_{Min} = −0.47 MPa and − 2.27 MPa in 2014 and

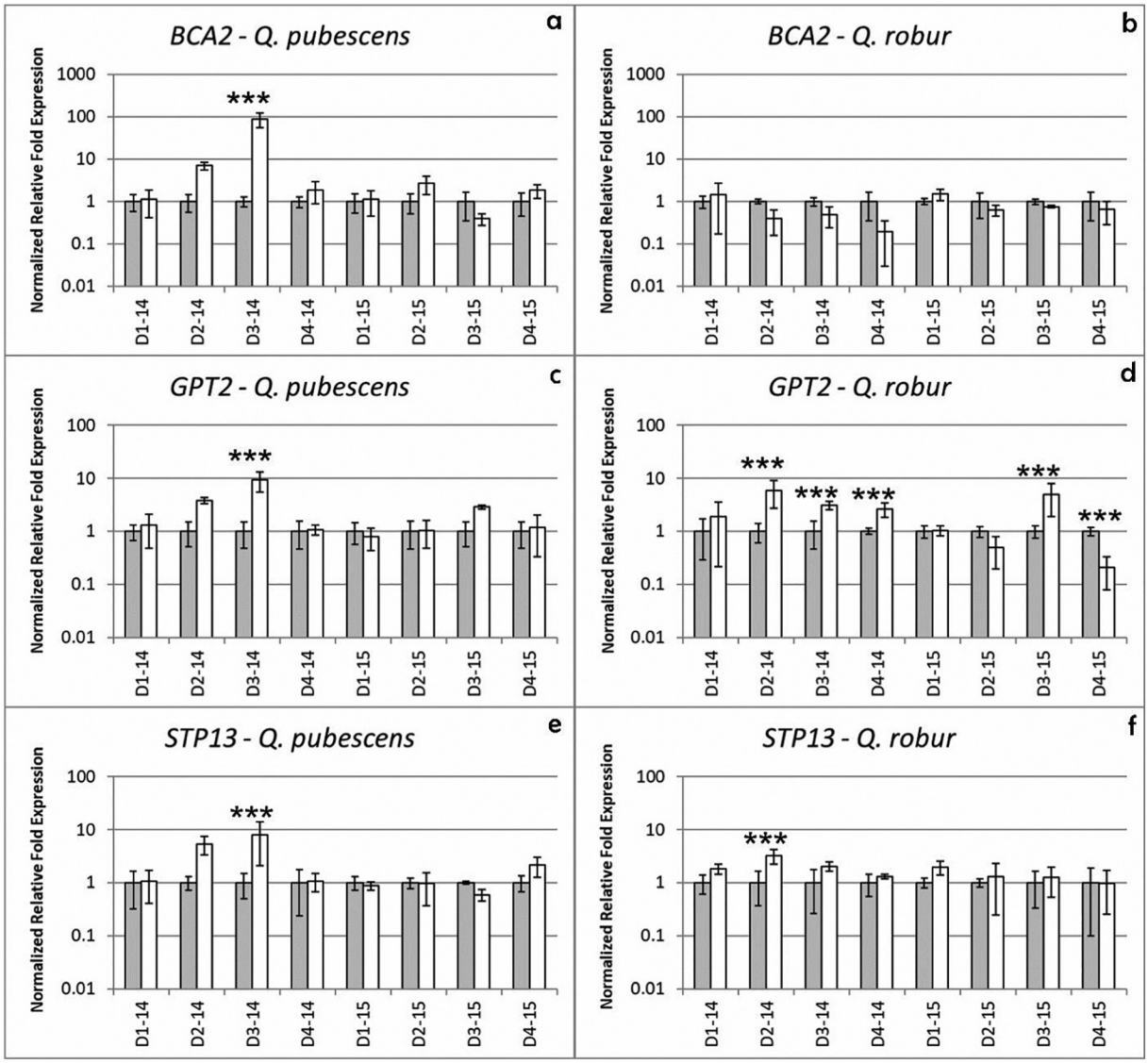

Fig. 3. a-f - Gene expression profiles of the genes BCA2, GPT2 and STP13 in Q. pubescens and Q. robur comprising the eight sampling dates D1–14 to D4–15 in 2014 and 2015. Gray bars: CO group; White bars: DS group; Significances are marked with asterisks (* = $p < .05$; *** = $p < .001$), error bars indicate standard error.

2015, respectively) could be an explanation for this finding. In *Q. robur*, however, the predawn water potential was similar in both years (−2.18 and − 2.27 MPa). Nevertheless, except for *RD22*, *NRX2* and *GPT2,* no significant gene regulation was observed at all in the second year of the drought experiment in *Q. robur*. Similar results were obtained in a study with a comparable experimental design using microarray technique to assess transcriptomic changes in *Q. robur* clones during a two-year drought experiment with repeated drought events (Spieß et al., 2012). In this study, 3% of the tested genes were differentially expressed, but 88% of these were differentially expressed on only one out of the eight sampling dates during the experiment. These findings can be explained as a constantly changing, very specific response pattern during the long-term response to drought. Furthermore, it has been reported that plants of many species, including *Quercus*, are able to memorize experienced stress and consequently alter their stress responses to repetitive drought events (Bruce et al., 2007; Galle et al., 2011; Fleta-Soriano and Munné-Bosch, 2016). Additionally, false positive results must be considered, e.g. caused by different genotypes or occasional gene regulation by drought unrelated events such as pathogen attacks.

4.3.1. ABA signaling pathway

The ABA dependent signaling pathway plays a dominant role in the drought stress response of plants (Vishwakarma et al., 2017). *PP2C27, RD22* and *STP13* have been identified as key molecules of the ABA signaling pathway in other plant species (Liu et al., 2012; Harshavardhan et al., 2014; Jia et al., 2017). Our results show that each of these three genes is clearly regulated differently in the two *Quercus* species and vary also between the two years of the experiment.

The upregulation of *STP13* was detected very early in both species although no further regulation was observed in the later course of the experiment under increased drought stress, thus indicating a role for *STP13* in the early stress response to mild drought. *STP13* is a high-affinity hexose transporter that is involved in sugar uptake and allocation (Schofield et al., 2009; Lemonnier et al., 2014) and is known to be drought responsive. Upregulation of *STP13* has been reported, inter alia, in the roots of cork oak (Magalhães et al., 2016). In leaves of poplar, *STP13* was identified to be a key molecule in a drought responsive hierarchical genetic network and in control of one of three subnetworks (Jia et al., 2017).

In contrast, *RD22* was upregulated in *Q. robur* on all dates with a decreased leaf water potential ($\Psi PD < -0.79$ MPa) in both years of the experiment, thus suggesting a role in the long-term response to sustaining drought stress. In the above mentioned comparable two-year drought experiment of Spieß et al. (2012) with *Q. robur*, upregulation of

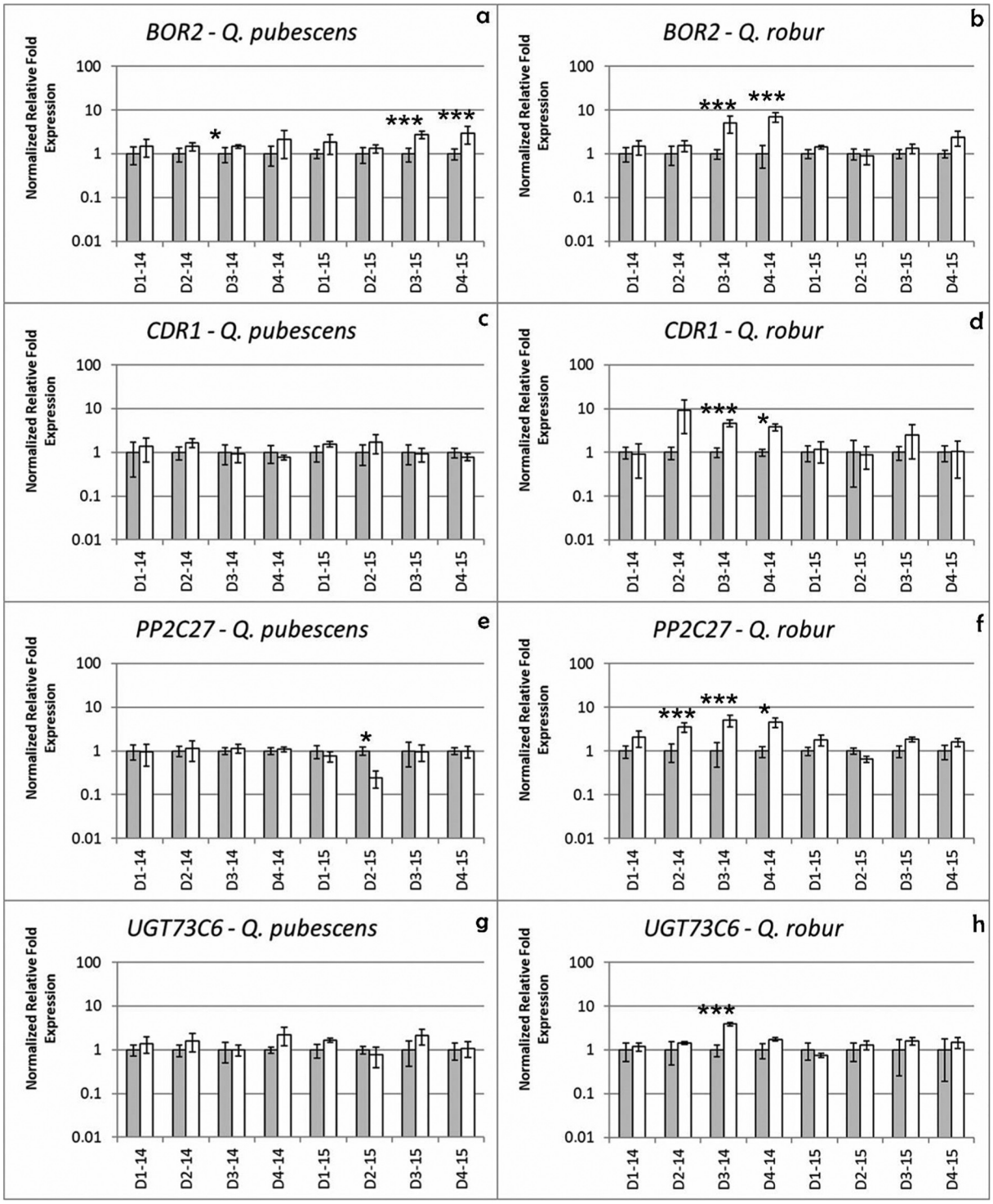

Fig. 4. a-h - Gene expression profiles of the genes BOR2, CDR1, PP2C27 and UGT73C6 in Q. pubescens and Q. robur comprising the eight sampling dates D1–14 to D4–15 in 2014 and 2015. Gray bars: CO group; White bars: DS group; Significances are marked with asterisks (* = $p < .05$; *** = $p < .001$), error bars indicate standard error.

RD22 was found on only one sampling date in the second year of the experiment. A similar expression pattern was found in this study in *Q. pubescens* where only under severe stress in the second year of the experiment (D3–15) was an upregulation found. The different expression patterns of *Q. robur* in the two studies may be explained by a higher stress intensity in this study while the different expression patterns between the species may reflect the enhanced drought stress tolerance of *Q. pubescens*. *RD22* is a key regulator of plant growth under drought stress; in *A. thaliana* it was shown that drought caused growth stalling faster in wildtype plants when compared to *RD22*-loss-of function mutants (Harshavardhan et al., 2014). A decreased shoot growth to save water and resources is a general strategy used by trees to survive drought events and is detectable earlier in drought sensitive than in tolerant species (Mooney et al., 2013; Brunner et al., 2015). Remarkably, *RD22* is discussed to have a function in drought stress memory and an ABA-independent induction of expression was

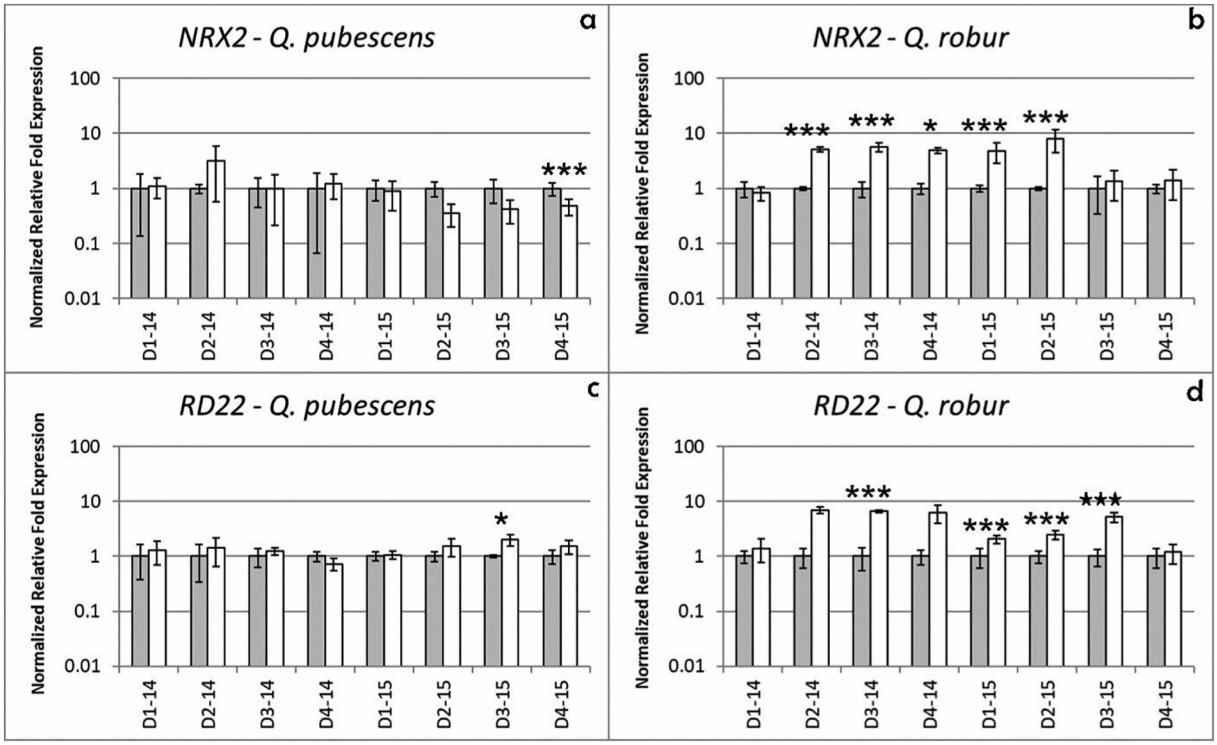

Fig. 5. a-d - Gene expression profiles of the genes NRX2 and RD22 in Q. pubescens and Q. robur comprising the eight sampling dates D1–14 to D4–15 in 2014 and 2015. Gray bars: CO group; White bars: DS group; Significances are marked with asterisks (* = $p < .05$; *** = $p < .001$), error bars indicate standard error.

demonstrated in *A. thaliana* (Goh et al., 2003).

PP2C27 was upregulated in response to drought stress in *Q. robur* only in the first year of the experiment, whilst and in *Q. pubescens* no upregulation was observed at all, although a downregulation under moderate drought stress was observed (D2–15). *PP2C27* was shown to induce the expression of drought responsive genes in *A. thaliana* (Liu et al., 2012) and the different regulation in the two *Quercus* species may reflect their different drought stress tolerance. The upregulation in *Q. robur* probably activates certain drought responsive genes, while the downregulation in *Q. pubescens* on D2–15 could be interpreted as a suppression of these genes under moderate drought stress that is later repealed (D3–15) under severe drought stress when the upregulation of *RD22* was also observed. The different gene expression of *PP2C27* in the two years in *Q. robur* could be explained by adjustment processes to repeated drought stress.

4.3.2. JA and ethylene pathways

It has been demonstrated that the increase of ABA concentration in the short-term response suppresses or promotes signaling networks of other phytohormones, such as JA or ethylene, in herbaceous plants (Yang et al., 2014). Furthermore, foliar ABA levels gradually decrease under persisting drought stress leading to a modified long-term drought stress response (Yang and Guo, 2007; Verslues, 2016; Kalladan et al., 2017). Therefore, in this study, genes associated with different phytohormone pathways were monitored to gain information on their role in drought stress responses in the two *Quercus* species.

The JA signaling pathway is a key regulator in pathogen defense and plays a role in the response to abiotic stresses including drought (Creelman & Mullet 1995; Wasternack and Parthier, 1997). The JA pathway can be activated by the upregulation of *AOS1* that is the major control point in JA biosynthesis (Laudert and Weiler, 1998). The upregulation of *AOS1* that was found in *Q. pubescens* in 2014 could be part of a response to mild DS, however, this explanation appears unlikely since the expression values kept increasing after rewatering. The JA signaling pathway was probably activated independently of drought stress, possibly by an unrecognized pathogen attack.

ERF4 is an ethylene responsive transcription factor and was selected as a target gene because it has been shown to be involved in the stress response by modulating the ethylene and ABA responses (Yang et al., 2005; Shinshi, 2008), while according to McGrath et al. (2005) *ERF4* forms part of the JA regulated pathogen defense response in *A. thaliana*. The latter role is supported by our results since the conspicuous similarity of the expression profiles of *AOS1* and *ERF4* in *Q. pubescens* indicates a drought stress independent coregulation. The ethylene and JA signaling pathways also play a crucial role in regulating leaf senescence (Kim et al., 2015), however, an upregulation of *AOS1* and *ERF4* due to senescence processes is unlikely since no upregulation was detected in *Q. robur* despite it showing premature senescence (Früchtenicht et al., 2018a).

4.3.3. Phenylpropanoid pathway, detoxification

PAL exhibits a similar gene expression profile to *AOS1* and *ERF4*. *PAL* is a key enzyme in the biosynthesis of phenylpropanoids and their derivatives such as flavonoids and lignins (Kong, 2015). In the comparative transcriptomics study of Madritsch et al. (2019, in revision), an enrichment of upregulated genes associated to the phenylpropanoid pathway was identified in *Q. robur* under severe drought stress, indicating that the pathway plays a role in drought stress response. Altered lignification and oxidative protection through quercetins and other flavonoids are known mechanisms of the drought response (Plomion et al., 2006; Nakabayashi et al., 2014) although *PAL* expression can also be induced by wounding and pathogens, as well as exogenous ethylene and JA (Guo and Wang, 2009; Kong, 2015). The activation of *AOS1* by JA could be the cause of the similar expression profiles of *PAL, AOS1* and *ERF4* in *Q. pubescens* in 2014. According to these findings, genes that were identified as differentially expressed in the comparative transcriptomics study and that are known to be responsive to JA should be handled with care, especially since *AOS1* and *ERF4* were demonstrated not to be drought responsive in *Q. robur*.

Regarding *PAL*, in *Q. robur* a downregulation was found in both

years of the experiment, whilst in *Q. pubescens* a non-significant downregulation was found under severe stress in the second year of the experiment. This finding suggests a downregulation of *PAL* under intense long-term drought stress. In other plant species, the response of *PAL* activity to ABA or drought stress varies; in *Populus cathayana* and maize an upregulation has been found (Xiao et al., 2009; Gholizadeh, 2011), while in tea and kenaf a downregulation was reported (Singh et al., 2009; Jeong et al., 2012) and in white clover an upregulation was reported in the short-term response and a downregulation in the long-term response after 14 days (Lee et al., 2007).

UGT73C6 plays a role in the biosynthesis of quercetins and other flavonoids (Jones et al., 2003; Lim et al., 2004) and was upregulated under severe drought stress in the first year of the experiment in *Q. robur*, but not in *Q. pubescens*. Quercetins and other antioxidant flavonoids are accumulated in *Quercus* (Rivas-Ubach et al., 2014) and other plant genera (e.g. Fini et al., 2012; Ma et al., 2014) in response to drought stress. The missing coregulation of *PAL* and *UGT73C6* indicates that the activation of the phenylpropanoid pathway observed in 2014 in *Q. pubescens* was not directed for the biosynthesis of quercetins.

Thioredoxins, such as *NRX2*, are part of the stress defense mechanisms of plants and contribute to stress tolerance against oxidative and drought stress (Cha et al., 2015; Kneeshaw et al., 2017). An upregulation of *NRX2* in response to drought stress was recently demonstrated in genetically modified *A. thaliana* which showed enhanced drought tolerance after inserting a dehydration-induced translation initiation factor from a drought tolerant soybean (Gallino et al., 2018). *NRX2* is upregulated in *Q. robur* from the onset of drought stress in the first year and the differential expression stops in the second year under severe stress. Interestingly, in the more drought tolerant *Q. pubescens*, there was no up-, but a downregulation of *NRX2* detected. The finding that both genes involved in protection against oxidative stress (*UGT73C6* and *NRX2*) are only upregulated in the less drought tolerant *Q. robur* is surprising since more drought tolerant genotypes are thought to express protectant enzymes to a higher degree (Wang et al., 2003). This finding might be simply explained by there being less oxidative stress in *Q. pubescens* due to its efficient competition for residual soil water with *Q. robur* (Früchtenicht et al., 2018a, 2018b).

4.3.4. Salicylic acid dependent defense response

CDR1 encodes an apoplastic aspartic protease which is a key regulator in the salicylic (SA) dependent defense response to biotic stress. Its overexpression leads to elevated SA and elevated defense response related protein levels in different plant species (Xia et al., 2004; Prasad et al., 2009). The gene is upregulated in *Q. robur* in the first year of the experiment, but not in *Q. pubescens*. Since the regulation of SA is part of the drought stress response in plants (Pandey and Chakraborty, 2015) and *CDR1* is known to be involved in regulation of SA levels, a role in drought stress response can not be excluded. Aspartic proteases that respond to drought and/or SA treatment are known in plants (Guo et al., 2013; Vantini et al., 2015).

4.3.5. Photosynthesis

Generally, photosynthesis and related anabolism is reduced in response to drought stress, though less drought tolerant genotypes reduce these processes earlier than more drought tolerant genotypes (Reddy et al., 2004).

The chloroplastic beta carbonic anhydrase *BCA2* probably supplies RuBisCO with carbon dioxide and has been linked to drought stress tolerance in several plant species (Moroney et al. 2001; Sun et al., 2016; Wang et al., 2016). *BCA2* probably enhances the water use efficiency of plants by elevating the carbon dioxide levels when stomata are closed. Another role for this gene has been discussed in the control of stomatal closure (Moroney et al., 2001). In *Q. pubescens* an upregulation of *BCA2* was monitored under mild drought stress whilst a trend of downregulation was visible in both species under severe drought stress, indicating that the expression of the gene is affected by the intensity of the stress and that an upregulation is part of the response to mild drought stress. In a study that assessed changes in the photosynthesis related leaf proteome of drought stressed *Q. robur*, a similar downregulation of a carbonic anhydrase was found (Sergeant et al., 2011).

GPT2 enables the uptake of glucose-6-phosphate into chloroplasts and is essential for the photosynthetic acclimation to changing environments (Flügge, 2002; Athanasiou et al., 2009). An upregulation in response to drought stress or ABA was reported in rice and *A. thaliana* (Peleg et al., 2011; Kim et al., 2011; Pandey et al., 2013). In *A. thaliana*, the expression of *GPT2* is under control of the ABA-responsive transcription factor *AREB1*, which is a key regulator of the ABA-dependent drought stress response (Sakuraba et al., 2015). In the drought stress response, increased *GPT2* levels probably lower stomatal conductivity by increasing the uptake of glucose-6-phosphate (the preferred substrate for starch biosynthesis in guard cells) to the chloroplasts. Starch synthesis in guard cells, in turn, induces stomatal closing (Prasch et al., 2015; Azoulay-Shemer et al., 2016). *GPT2* was strongly upregulated in both species in the first year of the drought experiment and to a lower extent in the second year of the experiment, possibly due to by an adjustment process.

4.3.6. Nutrient balance

Decreased water supply can cause nutrient deficits as a secondary effect (da Silva et al., 2011). For the micro-nutrient boron, it was demonstrated that foliar boron application can improve plant growth of crop plants under drought conditions (Hajiboland and Farhanghi, 2011; Abdel-Motagally and El-Zohri, 2018; Naeem et al., 2018). Boron transport had long been believed to be a passive process, but, upon boron transporters being identified, it has now been suggested that plants sense and respond to boron levels and regulate the expression of boron transporters to maintain homeostasis (Miwa and Fujiwara, 2010). *BOR2* encodes an efflux boron transporter that is localized in the plasma membrane; it is strongly expressed in root cells and is involved in root elongation (Miwa et al., 2013). The data from the recent drought experiment shows that *BOR2* is upregulated in the leaves of both oak species in response to drought stress. In *Q. pubescens*, *BOR2* was upregulated under mild drought stress in the first year and under severe stress in the second year of the experiment. In *Q. robur* it was upregulated only in the first year of the experiment. The missing differential expression in the second year may be caused by an adjusted gene expression in response to the repeated drought stress. The expression pattern when first experiencing severe drought stress is similar in both species.

4.4. Conclusion

Twelve representative DEGs were selected based on a comparative transcriptomics study in drought challenged *Q. pubescens* and *Q. robur*, for both their validation by RT-qPCR and for further expression analysis. Over the course of a two-year drought experiment with repeated long-term drought periods comprising eight sampling dates, expression profiles were generated for the selected genes. Despite only 12 genes being assessed, the results clearly show the complexity of the molecular response to repeated long-term drought stress. The gene expression pattern for one gene (*RD22*) had already been assessed in an earlier drought stress study in *Q. robur* (Spieß et al., 2012) and an upregulation in response to severe drought stress was confirmed in this study; the other eleven genes were studied in detail here for the first time. Each of the three genes involved in the ABA signaling pathway (*PP2C27, RD22* and *STP13*) was clearly regulated differently, giving an impression of the response pattern in the long-term drought response and showing the adjustment of gene expression to the repeated drought events. Two genes (*BOR2* and *UGT73C6*) were shown for the first time to be drought responsive. Three genes (*BCA2, NRX2, PP2C27*) showed a contrary regulation in the two species that may be linked to their different drought tolerance. Furthermore, three genes that were identified in the

Q. pubescens transcriptomics data set and that are associated with the JA signaling pathway (*PAL*, *AOS1* and *ERF4*) showed no correlation with the drought stress treatment, contradicting a contribution of the JA pathway to the modulation of the drought stress response.

To sum up, the quantification from the RNA-Seq was confirmed and detailed expression patterns for the selected genes were generated, contributing to the understanding of the molecular drought stress response in forest trees. The results also reveal the complexity of the drought stress response and show that much more effort must be expended for its full elucidation.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Detailed information on the RT-qPCR experiments according to the MIQE guidelines is provided in supplementary text File A. Detailed information on the target genes and the results of the calculations with Rest2009 is provided in supplementary Table 2. Supplementary data to this article can be found online at https://doi.org/10.1016/j.plgene.2019.100193

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