Whole-transcriptome response to water stress in a California endemic oak, *Quercus lobata*

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Reduced water availability during drought can create major stress for many plant species. Within a species, populations with a history of seasonal drought may have evolved the ability to tolerate drought more than those in areas of high precipitation and low seasonality. In this study, we assessed response to water stress in a California oak species, *Quercus lobata* Née, by measuring changes in gene expression profiles before and after a simulated drought stress treatment through water deprivation of seedlings in a greenhouse setting. Using whole-transcriptome sequencing from nine samples from three collection localities, we identified which genes are involved in response to drought stress and tested the hypothesis that seedlings sampled from climatically different regions of the species range respond to water stress differently. We observed a surprisingly massive transcriptional response to drought: 35,347 of 68,434 contigs (52%) were differentially expressed before versus after drought treatment, of which 18,111 were down-regulated and 17,236 were up-regulated. Genes functionally associated with abiotic stresses and death were enriched among the up-regulated genes, whereas metabolic and cell part-related genes were enriched among the down-regulated. We found 56 contigs that exhibited significantly different expression responses to the drought treatment among the three populations (treatment × population interaction), suggesting that those genes may be involved in local adaptation to drought stress. These genes have stress response (e.g., WRKY DNA-binding protein 51 and HSP20-like chaperones superfamily protein), metabolic (e.g., phosphoglycerate kinase and protein kinase superfamily protein), transport/transfer (e.g., cationic amino acid transporter 7 and K+ transporter) and regulatory functions (e.g., WRKY51 and Homeodomain-like transcriptional regulator). Baseline expression levels of 1310 unique contigs also differed among pairs of populations, and they were enriched for metabolic and cell part-related genes. Out of the large fraction of the transcriptome that was differentially expressed in response to our drought treatment, we identified several novel genes that are candidates for involvement in local adaptation to drought.

Keywords: drought, gene expression, local adaptation, *Quercus*, RNA-Seq.

Introduction

In plants, the capacity to respond to increases in temperature and drought conditions will be of particular importance if current climate trends continue (IPCC 2007, Diffenbaugh et al. 2015). Under most climate change scenarios, one of the main environmental stress factors will be increased drought (IPCC 2007, Dai 2013). In general, water availability is a major determinant of the distribution and abundance of plants. Understanding the mechanisms of how plants cope with water stress has been a central topic in plant physiology for decades (Stebbins 1952, Bray 1997). Many plants species have evolved specific adaptive mechanisms in response to the drought stress, exhibiting either...
drought escape or drought tolerance mechanisms (e.g., Savage and Cavender-Bares 2011). These mechanisms are complex, polygenic traits that involve cascades of responses, ranging from physiological changes to transcriptional regulation (Chaves et al. 2003). The key adaptive responses to drought in plants are reduced water loss, enhanced water uptake through roots and reduced radiation absorption. Physiological studies have shown that reduction in vegetative growth, stomatal closure and a decrease in the rate of photosynthesis are among the earliest responses to drought, protecting the plant from extensive water loss and oxidative stresses (Chaves et al. 2003). These drought response strategies and abilities can vary within species among different populations. For example, thick leaves and small stomata often found in oak species are characteristics that favor high water-use efficiency (Abrams 1990). Water-use efficiency, which is the ratio of the rate of photosynthesis to the rate of transpiration, is known to be important in local adaptation of oaks (Quercus spp.) (Ponton et al. 2002, Aranda et al. 2007, Ramirez-Valiente et al. 2009, Roussel et al. 2009).

With modern whole-transcriptome gene expression profiling (RNA-Seq) (Wang et al. 2009), it is feasible to study changes in gene expression controlled by water availability. Drought stress triggers many changes in gene expression that in turn affect a variety of physiological and metabolic processes, signal transduction, osmotic regulation and gene regulation in accordance with stress response (Shinozaki and Yamaguchi-Shinozaki 2007, Osakabe et al. 2014). Many of the genes involved in drought stress response have been identified as differentially expressed transcripts (Bray 2002, Ramanjulu and Bartels 2002, Osakabe et al. 2014). These genes can be categorized in two broad groups: those encoding stress response proteins versus regulatory proteins (Shinozaki and Yamaguchi-Shinozaki 2007). Stress response proteins include late embryogenesis abundant proteins or dehydrins, proteins involved in protecting photosynthesis, aquaporins that facilitate transmembrane water movement, lipid transfer proteins involved in cuticle biosynthesis, antioxidant proteins (e.g., glutathione-S-transferase) that reduce oxidative stress, carbohydrate transporters that adjust metabolism, osmolyte transporters to maintain osmotic balance, protein repair enzymes, proteases, protease inhibitors that block programmed cell death (e.g., Kunitz type) and other enzymes (Ramanjulu and Bartels 2002). Regulatory proteins of signal transduction and gene expression include transcription factors (e.g., WRKY, MYB, HD-ZIP, Myc-like bHLH), protein kinases, protein phosphatases, calmodulin-binding protein and those involved in abscisic acid biosynthesis (ABA), which can lead to stomatal closure, among other physiological phenomena (Seki et al. 2007). These types of proteins seem to be involved in immediate responses of plants to drought stress, but additional pathways can lead to long-term acclimation, damage control and repair, leaf senescence or programmed cell death (Osakabe et al. 2014). Furthermore, these responses and the molecules involved can vary within species depending on the genetic background of the individual, which may be related to local adaptation and different strategies or capacities for coping with drought (Villar et al. 2011, Lasky et al. 2014).

Quercus spp. (oaks) are known to display a wide range of adaptive variation for drought tolerance among species (Abrams 1990, Tyler et al. 2006) and among populations within species (Ponton et al. 2002, Roussel et al. 2009), but the molecular mechanisms underlying differences in tolerance are largely unknown. A few genes have been implicated in drought response in the European oak Quercus petraea based on a small expressed sequence tag (EST) study: betaine-aldehyde dehydrogenase, an ABA responsive transcription factor, an ABA-independent transcription factor, glutathione-S-transferase and a heat-shock cognate protein (Porth et al. 2005). In addition, a large EST microarray study of Quercus robur in response to long-term water stress showed that up-regulated genes encode protective proteins (e.g., late embryogenesis abundant, RCI2B, protein phosphatase 2CA), transcription factors (e.g., WRKY), transport proteins (e.g., inositol transporter 1 and lipid transfer proteins), leaf senescence proteins (ATP-dependent Cpl proteases and senescence-associated protein 21) and amylases (Spieß et al. 2012). Down-regulated genes were found to encode photosynthesis-related proteins. Finally, up to 20 genes with a broad range of functions were implicated in local adaptation to precipitation regimes in Quercus lobata Née in California based on extreme correlations of single-nucleotide polymorphisms with precipitation variables (Gugger et al. 2016, Sork et al. 2016). To assess the full array of genes that could be involved in drought adaptation, it is advantageous to use a whole transcriptome approach and avoid the ascertainment bias of studying only a few known genes. Additional knowledge of gene co-expression and the molecular pathways involved in adaptation of oaks to environmental changes will enhance our understanding of the molecular basis of adaptation and the capacity of oaks to recover under different frequencies, severities and durations of environmental stress (Spieß et al. 2012).

In this study, we take one approach to understanding plant gene expression response to water stress in Q. lobata, a widely distributed California oak found across a range of climate conditions. The distribution of this species is water-limited (McLaughlin and Zavaleta 2012), but its range includes a tremendous precipitation gradient (Sork et al. 2010). Thus, Q. lobata offers an excellent system to study tolerance and local adaptation in natural populations. Different populations might respond differently to projected increases in drought due to genetic differences, as well as differences in the severity of drought across the distribution. In this study, we assess the transcriptional response to drought stress by measuring gene expression profiles across the entire transcriptome. Specifically, we examine what genes are involved in response to drought stress and test the hypothesis that seedlings sampled from different climatic regions of
the species range respond to water stress differently, consistent with local adaptation.

Materials and methods

Sample design

The seed sources came from different maternal lines in each of three populations with contrasting climatic environments from across California: Malibu Creek State Park (MC); Middle Creek Campground, Mendocino National Forest (MK); and Springville (SV) (Figure 1 and Table S1 available as Supplementary Data at Tree Physiology Online). Springville is an open oak savanna ecosystem with the highest mean temperature and temperature seasonality and the lowest mean annual precipitation of the three sites, contributing to it having the highest climatic water deficit (CWD), which is an integrated measure of water availability including temperature, rainfall and soil characteristics (Flint et al. 2013). Middle Creek Campground is an open-canopy, mixed-oak woodland with the lowest CWD, highest precipitation and moderate temperature seasonality. Malibu Creek State Park is a mixture of oak savanna and mixed-oak woodland with CWD nearly as high as SV, the lowest temperature seasonality and low precipitation. In October 2011, acorns were collected from these three sites as part of a trial for a large provenance study of valley oak planted at the Institute of Forest Genetics, USDA Forest Service, Placerville, CA (Delfino Mix et al. 2015). Acorns were planted in Sunshine #4 (Sungro, Agawam, MA, USA) aggregate plus soil mix in Steuwe and Sons D40 pots (25 cm depth × 6.4 cm diameter) (Tangent, OR, USA) and watered twice per week and as needed to maintain moist soil. On 23 August 2012, mature leaf tissue samples (fourth node from top) of nine seedlings (three from each of three source populations) were collected and frozen immediately between slabs of dry ice for RNA isolation to be used as control samples before treatment. To simulate a water stress condition, these same seedlings were deprived of water for 15 days, after which (7 September 2012) we collected tissue on dry ice for RNA isolation.

Library preparation and sequencing

Total RNA was isolated with a prewash protocol (http://openwetware.org/wiki/Conifer_RNA_prep) and RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality and quantity of the total RNA was determined using a BioAnalyzer 2100 with RNA Nano chip (Agilent Technologies, Santa Clara, CA, USA). We then prepared cDNA from poly-A purified mRNA for 50-bp, single-end sequencing using the Illumina TruSeq RNA library preparation kit. Each sample was barcoded using standard Illumina adaptors 1–12 to allow up to 12 samples to be pooled in each of two lanes of sequencing on an Illumina HiSeq 2000 v3 at the Broad Stem Cell Research Center, UCLA. A given before–after pair was sequenced on the same lane, and sample pairs drawn from different populations were stratified across the two sequencing lanes (see Table S1 available as Supplementary Data at Tree Physiology Online).

Processing and mapping Illumina reads

The quality and contamination levels of RNA-Seq reads generated by the Illumina HiSeq 2000 were ascertained using FastQC. Low quality reads, primer/adapter contamination, and long repetitive sequences were filtered using tools on the UCLA Galaxy server (http://galaxy.hoffman2.idre.ucla.edu/). Filtered reads were aligned against all contigs in the reference transcriptome available for Q. lobata (Cokus et al. 2015) using BWA, the Burrows–Wheeler Aligner with default settings (Li and Durbin 2009, 2010). Burrows–Wheeler Aligner uses an index built with the Burrows–Wheeler transformation that allows for fast searching and also reports a meaningful mapping quality score that can be used to discard alignments that are not well supported due to a high number of mismatches or low mapping quality. The numbers of reads mapped to each contig were generated using Samtools 0.1.19 ‘Gene to Counts’ (idstats) (Li et al. 2009) and used for subsequent analyses. Approximately 23 k of 83 k contigs in the Q. lobata reference transcriptome (Cokus et al. 2015) have gene models assigned to them, of which 9.4 k were assigned functional annotations based on Arabidopsis orthologs (Swarbreck et al. 2008) and 19 k were assigned annotations based on matches to the Pfam protein family database (Finn et al. 2014).
**Identification of differentially expressed genes**

Differential expression before versus after drought, differences in baseline expression levels among populations, as well as differential response to drought by different populations were analyzed using gene lengths and count data in DESeq2 1.6.3 (Anders and Huber 2010, Love et al. 2014) as implemented in R 3.1.2 (R Development Core Team 2014) and the R Bioconductor project (http://www.bioconductor.org). DESeq2 implements a negative binomial generalized linear model utilizing size-factor normalized gene expression data (Anders and Huber 2010). Our final model included ‘treatment’ (before vs after drought), sequencing lane, library prep batch, population and population × treatment interaction as predictors (see Table S1 available as Supplementary Data at Tree Physiology Online) and expression data for all contigs as response variables. We ran this model with Wald tests and extracted the relevant contrasts (e.g., before versus after, and interactions). Contigs with low expression across most samples for a given contrast are automatically discarded from testing in DESeq2. Resulting P-values were adjusted for multiple testing using the Benjamini and Hochberg (1995) false discovery rate (FDR) method, and 0.05 was used as a threshold for significance. Based on regularized log-transformed expression count data, principal components analysis and hierarchical clustering with heatmaps were used to visualize the results.

To arrive at our final DESeq2 model (above), we considered simpler models that included expression data only at contigs with gene models or excluded ‘lane’ and ‘library prep’ terms. The results were highly similar, thus we have preferred to include all contigs and follow the common practice of accounting for batch effects. Furthermore, the DESeq2 modeling framework does not allow for a term for ‘individual’ to be included, thus we assessed the potential for bias in our model by estimating the intra-individual correlation based on mixed models with individual, lane, library prep batch, population and treatment as random effects, as implemented in the variancePartition package in R 3.3.1 using regularized log-transformed expression data. The median variance explained for the ‘individual’ term across all genes, which can be interpreted as the intra-individual correlation, is equal to zero (see Figure S1 available as Supplementary Data at Tree Physiology Online), suggesting that individual variation is unlikely to create spurious results favoring our hypotheses.

**Gene ontology enrichment analyses**

We performed singular enrichment analyses using agriGO (Du et al. 2010) to identify functional classes of genes based on Plant Gene Ontology (GO) slim (Ashburner et al. 2000) annotations drawn from the Arabidopsis Information Resource (TAIR10, http://www.arabidopsis.org; Swarbreck et al. 2008) that are overrepresented in the lists of significantly differentially expressed genes relative to a background of all genes that were tested in DESeq2. Specifically, we performed Fisher’s exact tests for GO terms that occurred in at least 10 genes and corrected for multiple testing using the FDR method of Benjamini and Yekutieli (2001). A threshold of 0.05 was used to assess significance based on the FDR-adjusted P-values. This analysis was necessarily restricted to the set of 9.4 k oak contigs for which Arabidopsis orthologs had been assigned (Cokus et al. 2015).

**Weighted gene co-expression network analysis**

To construct a gene co-expression network, we deployed WGCNA 1.51 (Langfelder and Horvath 2008) using biweight midcorrelation among normalized gene expression values based on the regularized log-transformation as implemented in DESeq2. We considered only genes with expression level counts ≥10 in at least 90% of libraries. Groups of genes with similar expression patterns (‘modules’) were identified based on hierarchical clustering (soft threshold power = 7, minimum module size = 100, 0.1 ≤ mergeCutHeight ≤ 0.3). The module eigengenes (i.e., the first principal component of the module) were associated with the main drought stress treatment (as dummy variable) and geographic and climatic variables from the sample site of origin using linear regression (Zhang and Horvath 2005). Climate data included growing season growing degree-days above 5 °C derived from a spline model (Rehfeldt 2006), and CWD (a measure of evaporative demand exceeding soil moisture), actual evapotranspiration, mean maximum temperature (Tmax) and mean minimum temperature (Tmin) derived from the California Basin Characterization Model 1950–1980 averages (see Table S1 available as Supplementary Data at Tree Physiology Online) (Flint et al. 2013). All WGCNA settings were set at their default values unless previously mentioned. An advantage of the WGCNA approach is that it can identify groups of putatively interacting genes independent of preexisting annotation information.

**Results**

**Gene expression and GO enrichment**

In total, nine individuals in three populations of Q. lobata were sequenced successfully across both experimental conditions (before and after drought stress) (see Table S1 available as Supplementary Data at Tree Physiology Online). A range of 6.5–24.7 million (mean = 13 million) 50-base reads were obtained for each treatment of each biological sample after filtering (NCBI BioProject <TBD>). Of these, 77% of filtered RNA-Seq reads mapped onto the Q. lobata reference transcriptome (unaligned reads were discarded).

Surprisingly, 35,347 of 68,434 contigs with sufficient expression levels for testing (52%) were significantly differentially expressed before versus after drought treatment (Figure 2a and Table S2 available as Supplementary Data at Tree Physiology Online). Of these, 18,111 were down-regulated and 17,236 were up-regulated in response to drought (Table 1, Figure 2 and Table S2 available as Supplementary Data at Tree Physiology Online).
Online). Of these genes, 91% had greater than twofold change in expression before versus after the drought treatment. This large response is also apparent in a principal components analysis showing that samples collected before treatment cluster together and those after treatment cluster together (Figure 3). Genes up-regulated in response to the drought treatment are involved especially in responses to stimulus, response to stress, death and various nuclear cellular components (Figure 4a and Figure S2 available as Supplementary Data at Tree Physiology Online). Down-regulated genes primarily have functions related to photosynthesis, metabolic processes and various cell parts (Figure 4b and Figure S3 available as Supplementary Data at Tree Physiology Online). Given the very large number of genes that responded to the treatment, we also performed an additional enrichment analysis restricted to the 3000 most significant genes. The results were largely the same (not shown). A number of candidate genes identified in other studies are also differentially expressed in ours (see Discussion).

Fifty-six contigs had different expression responses to the treatment depending on the population (population x treatment interaction) (Tables 1 and 2, and Figure 2b). A dendrogram and heatmap generated based on regularized log-transformed expression levels reveal two clusters of genes with similar interaction patterns (Figure 5). The clusters seem unrelated to any functional type of gene (e.g., metabolic genes are in both clusters). These 56 genes include eight with Arabidopsis orthologs: a WRKY transcription factor (m01oak15323cc-t01.1, AT5G64810), an ATPase family gene (m01oak16888Cc-t01.1, AT1G67120), HSP20-like chaperones superfamily protein (m01oak35262Cc-t01.1, AT4G10250), phosphoglycerate kinase (m01oak07301CC-t01.1, AT1G79550), protein kinase superfamily protein (m01oak13924Cc-t01.1, AT2G23200) and SPFH/Band 7/PHB domain-containing membrane-associated protein family (m01oak14227Cc-t01.1, AT2G03510). Of the remaining 49 genes, 21 had no known function and 28 contained Pfam domains with a broad range of molecular functions, especially related to stress response, regulation of metabolism, molecule transport or transfer, and nucleic acid binding and signaling. Due to the small number of TAIR-annotated genes, we were unable to statistically test for enrichment of GO functions.

![Figure 2](image-url)  
Figure 2. Log2-fold gene expression change versus normalized expression level for (a) test of before versus after the drought stress treatment and (b) test of the drought treatment by population interaction. Red points are statistically significant after FDR adjustment.

![Figure 3](image-url)  
Figure 3. Principal components analysis of regularized log-transformed gene expression data for the 500 most variable genes colored by sample site. All samples before drought treatment are clustered on the left and after are clustered on the right. MC, Malibu Creek State Park; SV, Springville; MK, Middle Creek.

<table>
<thead>
<tr>
<th></th>
<th>Total contigs</th>
<th>Tested in DESeq2</th>
<th>Differentially expressed</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before vs after</td>
<td>83,645</td>
<td>68,434</td>
<td>35,347</td>
<td>17,236</td>
<td>18,111</td>
</tr>
<tr>
<td>(‘treatment’)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before vs after</td>
<td>9431</td>
<td>9234</td>
<td>6768</td>
<td>3129</td>
<td>3639</td>
</tr>
<tr>
<td>(TAIR only)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Population x treatment</td>
<td>83,645</td>
<td>36,254</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malibu vs Middle Creek</td>
<td>83,645</td>
<td>32,392</td>
<td>537</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malibu vs Springville</td>
<td>83,645</td>
<td>28,277</td>
<td>767</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Springville vs Middle Creek</td>
<td>83,645</td>
<td>40,324</td>
<td>329</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Counts of differentially expressed genes based on DESeq2 during a 15-day simulated drought experiment for three populations of Q. lobata sampled in California.
Whole-transcriptome response to water stress in oak

Figure 4. Results of singular enrichment analyses based on Plant GO slim functional categories for genes that were significantly (a) up-regulated and (b) down-regulated in response to the drought treatment.

Baseline differences in gene expression levels among populations were also apparent: 530 genes differed in expression among MC vs MK, 752 genes among MC vs SV and 310 genes among SV vs MK (Table 1 and Table S3 available as Supplementary Data at Tree Physiology Online). Gene Ontology enrichment in these genes was primarily in categories unrelated to stress, such as photosynthesis, metabolism and cellular parts (Figure 6 and Figure S4 available as Supplementary Data at Tree Physiology Online).

**Weighted gene co-expression network analysis**

Weighted gene co-expression network analysis based on 17,601 genes with sufficient expression revealed two large modules of co-expressed genes (Figure 7). The first (‘grey’) module is composed of 3270 genes, and its eigengene has moderate, insignificant correlations ($0.1 < |r| < 0.37; P > 0.1$) with the climate of origin and virtually no correlation with the drought treatment (Figure 8, and Figures S5 and S6 available as Supplementary Data at Tree Physiology Online). The second (‘black’) module is composed of 14,331 genes whose eigengene is strongly associated with the drought treatment ($r = 0.96, P < 7 \times 10^{-10}$). Thus, the genes can be broken down as one very large group that responded to the drought treatment synchronously and another fairly large group that did not respond to the drought but may have modest differences in baseline expression level among sample sites or individuals. The coarse resolution of clusters may be due to relatively low sample size.
Table 2. Genes or contigs with significant differential expression for the drought treatment x population interaction.

<table>
<thead>
<tr>
<th>Quercus gene or contig</th>
<th>Arabidopsis TAIR or Pfam ID</th>
<th>Symbol</th>
<th>Protein or domain name</th>
<th>General function</th>
</tr>
</thead>
<tbody>
<tr>
<td>m01oak00470CT-t01.1</td>
<td>D:PF02969, D:PF00125</td>
<td>TAF Histone</td>
<td>TATA box binding protein associated factor (TAF), Core histone H2A/H2B/H3/H4</td>
<td>Metabolic</td>
</tr>
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<td>m01oak01092CC-t01.1</td>
<td>F:PF02225, D:PF00082</td>
<td>PA Peptidase_S8</td>
<td>PA domain, Subtilase family</td>
<td>Metabolic</td>
</tr>
<tr>
<td>m01oak03031CT-t01.1</td>
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<td>2 x Jacalin</td>
<td>Jacalin-like lectin domain</td>
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</tr>
<tr>
<td>m01oak03252cf</td>
<td>constrained</td>
<td></td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>m01oak05037Cz</td>
<td>constrained</td>
<td></td>
<td>Unknown</td>
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<td>m01oak05736Cz</td>
<td>constrained</td>
<td></td>
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<td>Unknown</td>
</tr>
<tr>
<td>m01oak05832Cm</td>
<td>constrained</td>
<td></td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
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<td>F:PF01764</td>
<td>Lipase_3</td>
<td>Lipase (class 3)</td>
<td>Metabolic</td>
</tr>
<tr>
<td>m01oak06325Ct-t01.1</td>
<td>F:PF03767</td>
<td>Acid_phosphat_B</td>
<td>HAD superfamily, subfamily IIIB (Acid phosphatase)</td>
<td>Metabolic</td>
</tr>
<tr>
<td>m01oak06417Ct-t01.1</td>
<td>F:PF03169</td>
<td>OPT</td>
<td>OPT oligopeptide transporter protein</td>
<td>Transport/transfer</td>
</tr>
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<td>PGK</td>
<td>Phosphoglycerate kinase</td>
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<td></td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
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<td>Methyltransf_7</td>
<td>AM-dependent carboxyl methyltransferase</td>
<td>Transport/transfer</td>
</tr>
<tr>
<td>m01oak09862Cf-t01.1</td>
<td>D:PF13561, F:PF08659,</td>
<td>adh_short_C2, KR,</td>
<td>Enoyl-(Acyl carrier protein) reductase, KR</td>
<td>Metabolic</td>
</tr>
<tr>
<td>m01oak12499Cz</td>
<td>D:PF00210</td>
<td>Ferritin</td>
<td>Ferritin-like domain</td>
<td>Ion homeostasis</td>
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<td>Protein kinase superfamily protein</td>
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<td>Unknown</td>
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<tr>
<td>m01oak15142CC-t01.1</td>
<td>r:PF00560</td>
<td>LRR_1</td>
<td>Leucine Rich Repeat</td>
<td>Protein binding</td>
</tr>
<tr>
<td>m01oak15323Cc-t01.1</td>
<td>AT5G64810</td>
<td>WRKY51</td>
<td>WRKY DNA-binding protein 51</td>
<td>Stress response, transcription factor</td>
</tr>
<tr>
<td>m01oak16669CC</td>
<td>AT1G67120</td>
<td></td>
<td>Unknown</td>
<td>Transcription factor, stress response</td>
</tr>
<tr>
<td>m01oak16888Chi-t01.1</td>
<td>AT1G67120</td>
<td></td>
<td>ATPases; nucleotide binding; ATP binding; nucleoside-triphosphatases; transcription factor binding</td>
<td>Stress response, transcription factor</td>
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<td>m01oak17139CC-t01.1</td>
<td>F:PF01657</td>
<td>2 x Stress-antfung</td>
<td>Salt stress response/antifungal</td>
<td>Stress response, pathogen response</td>
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<td>m01oak17816Ct-t01.1</td>
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<td>CAT7</td>
<td>Cationic amino acid transporter 7</td>
<td>Transport/transfer</td>
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</tr>
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Discussion

Massive response to drought

A striking result of this study is the massive gene expression changes observed in response to the drought treatment: 35,347 of 68,434 contigs tested, or 52%, were differentially expressed, most of which (91%) exhibited greater than twofold change in expression level (Figure 2a and Table S2 available as Supplementary Data at Tree Physiology Online). This result was even more drastic when considering only the highest quality contigs with assigned Arabidopsis orthologs: 6768 of 9234 genes or 73% (Table 1). Even in attempting to define subsets of genes with similar co-expression patterns, we found that genes could only be grouped into a large set that responded synchronously to the treatment (most genes) versus those that did not (Figure 7). Several studies that used similar approaches have reported responses involving up to 20–37% of the genes analyzed (Cohen et al. 2010, Bhardwaj et al. 2015), but most report <15% (e.g., Kreps et al. 2002, Utsumi et al. 2012, Yates et al. 2014). The extremely large gene expression responses to short-term drought provides many candidate genes for involvement in oak response to water stress, but it also likely includes other types of associated responses.

There are at least four potential non-mutually exclusive explanations for the large response to drought. One possibility is that gene expression varies dramatically through time, largely unrelated to water stress, and thus a large component of the apparent response stems from developmental or other changes that occurred within individuals during the 15-day interval pre- and post-treatment samples. Time-series experiments often identify many genes whose expression differs through time (usually in relation to development or stress), but typically the response is not as large as that observed here (e.g., Breeze et al. 2011, Bechtold et al. 2016). In addition, we attempted to minimize effects of development by collecting tissue from leaves of similar maturity at the same time of day. A second explanation is that the treatment was so severe that the plants were in the process of dying and shutting down expression altogether. In this scenario, we would expect the plants to show bias toward down-regulation of all genes after treatment; however, we observe similar fractions of genes up- and down-regulated (Table 1 and Figure 2, and see Table S2 available as Supplementary Data at Tree Physiology Online). A third, more likely, explanation is that our drought treatment was severe enough that pathways unrelated or indirectly related to drought, such as those related to leaf senescence, were initiated. Indeed, several leaves began browning around the time of collection, death-related genes were up-regulated after the treatment, and a number of non-drought related genes were differentially expressed (Figure 4), although this latter observation is common in RNA-Seq differential expression studies. Fifteen days without water occurs frequently in nature, and seedlings must routinely survive such stresses. However, the importance of acclimation in
stress response is widely reported in the plant physiology literature (Yordanov et al. 2000), and thus it is possible that this seemingly realistic drought treatment imposed on unacclimated greenhouse plants was ‘felt’ severely. A fourth possible explanation is that such a massive transcriptomic response is normal in oaks, which would indicate a flexible and dynamic response to the drought-prone environments that oaks often inhabit. In this case, we might expect that other species that occupy water-limited environments might also exhibit large responses. Alternatively, large responses may be a feature of all trees or some other aspect of the natural history of oaks. However, we do not observe either trend in other studies (Kreps et al. 2002, Cohen et al. 2010, Utsumi et al. 2012, Yates et al. 2014, Bhardwaj et al. 2015), and the response does not seem to be large (3%) in other oaks under long-term water stress (Spieß et al. 2012). The distinction between the latter two hypotheses for explaining the massive response (severe treatment versus real response) in valley oak can be better understood in future studies using data collected from seedlings in nature or additional greenhouse studies allowing for acclimation.

The massive response precludes us from pinpointing the precise molecular mechanisms of drought stress response, but the observed enrichment for a number of gene functional categories related to abiotic stress responses are similar to those found in Arabidopsis and other plants (Figure 4). Among those genes are many that are commonly implicated in drought response in oaks and other species, such as late embryogenesis abundant proteins, glutathione-S-transferase, zeaxanthin epoxidase (involved in ABA biosynthesis), chitinase family proteins, inositol monophosphatase family proteins, dehydration-induced proteins, several proteins that interact with ABA under drought, heat-shock proteins, lipid transfer proteins, and various transcription factors including WRKY32, WRKY57, MYB homeodomain-like superfamily proteins and bHLH nucleotide binding proteins (see Table S2 available as Supplementary Data at Tree Physiology Online) (Porth et al. 2005, Seki et al. 2007, Shinozaki and Yamaguchi-Shinozaki 2007, Spieß et al. 2012). Consistent with research in other oaks, we further find that senescence-associated and cell death-associated proteins are up-regulated and photosynthesis-related proteins are down-regulated (Spieß et al. 2012). In addition, genes whose patterns of nucleotide polymorphism implicate them in adaptation to local climate in Q. lobata (Gugger et al. 2016) are also enriched among the large set of genes responding to the drought treatment (40 of 50 climate-related genes from Gugger et al. are found differentially expressed genes from this study; hypergeometric test: \( P < 10^{-4} \)), lending further support to their role in climate stress adaptation.

Local adaptation to drought

We find initial evidence of local adaptation to drought in the different responses of the different sampled populations to the drought stress treatment. Specifically, the expression response of 56 genes to the drought treatment depended on population of origin (treatment \( \times \) population interaction) (Table 1, and Figures 2b and 4). Based on limited available information, these genes are not known to be involved in local climate adaptation in oaks (Gugger et al. 2016, Sork et al. 2016) nor Arabidopsis (Hancock et al. 2011), but have plausible functions for such involvement. These genes primarily function in metabolic processes, stress response, transport/transfer of other molecules, signaling and transcription regulation. In particular, HSP20-like chaperones superfamily protein is known to be involved in stress response and ‘memory’ (Stief et al. 2014), and other heat-shock proteins have been associated with water stress response in other oaks (Porth et al. 2005). In addition, phosphoglycerate kinase is involved in energy metabolism under stress (Larkindale and Vierling 2008) and protein kinase superfamily protein is involved in metabolic processes. Given the prevalence of molecules involved in metabolism (13 of 29 ascribed general functions), we hypothesize that local adaptation to drought in oaks may include different abilities to metabolize or different ways of altering metabolic activities in the face of drought. These different metabolic responses may be mediated by some of the
transcription factors among the list. These include: WRKY51, which is involved in regulating stress response to reduce reactive oxygen species and cell death (Rushton et al. 2010, Gao et al. 2011); an ATPase family gene involved in transcription regulation and stress response, possibly in an auxin signaling pathway (He et al. 2005); a homeodomain-like transcriptional regulator; or the proteins containing domains involved in signaling, such as m01oak50236t-t01.1 (Zn knuckle), m01oak29709cF-t01.1 (TIR domain) and m01oak18171VT- t01.1 (NB-ARC domain and leucine-rich repeats). Therefore, we speculate that, collectively, these molecules may be related to the commonly observed differences in water-use efficiency among populations of oaks (Aranda et al. 2007, Roussel et al. 2009).

Generally, we observed two clusters of genes that have similar drought treatment by population expression responses (Figure 5): one (lower half) corresponding to up-regulation in response to drought in MK and SV and modest up-regulation to down-regulation in the MC, and another (upper half) containing a number of population-specific patterns (Figure 5). Very few of these genes (e.g., m01oak15323cc, WRKY51; m01oak14227Cc, SPFH/ Band7/PHB domain-containing membrane-associated protein.

Figure 6. Results of singular enrichment analyses based on Plant GO slim functional categories for genes that were differentially expressed among sample sites.

Figure 7. Hierarchical clustering dendrogram of regularized log-transformed gene expression and inferred modules of co-expressed genes (gray versus black) based on WGCNA.

Figure 8. Heatmap of correlations (P-value in parentheses) of module eigengenes with the drought treatment, sample site coordinates and climate variables at the sample sites. Black and gray modules are as in Figure 7. GSDD5, growing season growing degree-days above 5 °C; AET, actual evapotranspiration; CWD, climatic water deficit; Tmin, mean annual minimum temperature; and Tmax, mean annual maximum temperature.
family) seem to follow a pattern that would suggest that high water stress sources (SV, MC) differ in response from low water stress sources (MK) (see Table S1 available as Supplementary Data at Tree Physiology Online), nor do they suggest a coast-interior divide or north–south divide as has been observed in genetic studies (Gugger et al. 2013, 2016). However, three seed sources are not sufficient to make robust inferences. These patterns of co-expression raise the possibility that tight interactions exist among the molecules in each cluster. Unfortunately, none of the eight oak genes assigned Arabidopsis orthologs are known or predicted to interact in Arabidopsis (Geisler-Lee et al. 2007), and such information is entirely lacking for oaks.

Baseline levels of expression in 1310 genes differed among populations in this study. These results do not inherently indicate anything about drought response, but fixed population differences in expression levels can contribute to local adaptation more generally. These genes tended to be involved in metabolism and cell parts (Figure 6), further implicating metabolic differences in population differentiation. These genes were not enriched among genes in the WGCNA ‘grey’ module, whose eigengene has moderate but insignificant correlations with the sample sites and their climates and has no correlation with the drought treatment (Figure 8). Nonetheless, each result independently offers lists of candidate genes for understanding differentiation among populations of Q. lobata.

In conclusion, this study provides initial evidence that seedlings from different localities and environments respond differently in gene expression, consistent with predictions based on local adaption. Given these differential responses to the treatment among populations and the different baseline expression levels among populations, we expect that different regional populations will have different responses and different capacities to respond to rapid climate change. This idea is consistent with inferences from ecological niche models suggesting differential responses among different regions (Sork et al. 2010) and a long history of provenance tests in trees suggesting local adaptation to environment and different tolerances for novel environments (Rehfeldt et al. 1999, Savolainen et al. 2007).

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Supplementary Data

Supplementary Data for this article are available at Tree Physiology Online.

Conflict of interest

None declared.

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