Title
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Water stress treatment in valley oak (*Quercus lobata*) seedlings reveals species-wide similarities

and population-specific differences in ecophysiological and gene expression response

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Biology

by

Alayna Mead

2017

ABSTRACT OF THE THESIS

Water stress treatment in valley oak (*Quercus lobata*) seedlings reveals species-wide similarities

and population-specific differences in ecophysiological and gene expression response

by

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Master of Science in Biology

University of California, Los Angeles, 2017

Professor Victoria Sork, Chair

Drought is a major stress for plants, and creates strong selection pressure for adaptive responses.
Many drought responses will be conserved, species-wide responses, but when populations are
distributed across heterogeneous environments, local selection pressures may shape differences in
their response to drought. This study tests whether populations of valley oak (*Quercus lobata*), a
widely-distributed California endemic oak, are locally adapted in their response to water stress.
Using groups of seedlings sampled from dissimilar climates and exposed to soil-drying or high
water treatments, we measured ecophysiological traits and gene expression (RNA-seq) data.
Valley oak seedlings under water stress had a lower leaf water potential and turgor loss point, but
populations were not significantly different from each other, indicating a generalized species-wide

response. However, most genes that were differentially expressed between treatments responded in only one seedling population, indicating that populations generally have different responses to water stress. Additionally, gene modules (groups of genes with similar expression patterns, identified using weighted gene co-expression networks) often responded differently to water stress treatment among populations, potentially identifying differences in drought response that occur through differential regulation of gene networks. This study provides evidence that valley oak populations are locally adapted to respond to water stress. As drought is projected to increase in California due to climate change, this may be useful for predicting the response of different populations and devising management strategies.

The thesis of Alayna Mead is approved.

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Acknowledgements

Introduction

Drought is a major abiotic stress for plants. Once plants become established, there is enormous selection pressure for the ability to survive in that site. Water is a particularly important resource because plants are continuously losing water through the stomata in order to photosynthesize (Bray 1997). In populations with high seedling mortality due to drought, selection pressure favors individuals that are adapted to the water availability of their local environment. This climate-related selection pressure is particularly relevant for tree species, as they tend to be long-lived and there is likely to be strong selection for surviving climate variations throughout their lifespan (Sork et al. 2013). Drought response strategies may be classified along a spectrum from drought avoidance, in which plants are able to maintain their water status under water stress by minimizing water loss; and drought tolerance, in which plants are able to survive and recover from water stress (Chaves et al. 2003; Juenger 2013). Broadly, plant drought responses may include stomatal closure, reduction of growth and photosynthesis, responses to reactive oxygen species, accumulation of solutes, and changes in sugar metabolism (Chaves et al. 2003; Seki et al. 2007; Pinheiro and Chaves 2011; Osakabe et al. 2014).

Specific drought responses may occur in an individual due to species-wide traits, genetic differences among populations, and phenotypic plasticity. Species-wide responses are often present due to fundamental ancestral genes that have been conserved due to strong purifying selection. These responses are likely to be important generalized drought responses that were present early in the evolution of the species, and may be shared among related species as well. Because species-wide responses are often shared due to the common descent of alleles that are

maintained in the species through negative selection, they can be defined as responses resulting from the same underlying genes (parallel adaptation involving different genes may also be possible, but is unlikely to occur in all populations of a species). Population differences in response, on the other hand, may arise through local adaptation to the population's climate, which can be formally defined as the pattern in which a genotype has higher fitness in its local climate than any other genotypes (Kawecki and Ebert 2004). This can occur when a species occupies a heterogeneous climate and consists of multiple populations with restricted gene flow. Phenotypic plasticity, the ability of a genotype to show different phenotypes in different environments, can be a heritable trait (Bradshaw 1965) that may be either species-wide or locally adapted; all adaptive stress responses are a form of phenotypic plasticity. Populations may vary in the degree of their plasticity, as it may be costly to maintain (DeWitt et al. 1998); so it is likely that variable environments will select for more plastic traits (Scheiner 1993). For example, an environment with frequent periodic droughts may select for greater plasticity in order to take advantage of wet periods and survive in dry periods.

Several ecophysiological traits can be used to characterize the drought response of plants (Lenz et al. 2006; Bartlett et al. 2012a; Mitchell and O'Grady 2015). Leaf water potential (Ψ_{leaf}) measures the water status of a plant, with a lower (more negative) value indicating more solutes in the leaves. All else being equal, plants under more severe water stress have a lower leaf water potential (Lenz et al. 2006). The osmotic potential at turgor loss point (π_{TLP}), or the wilting point, can be used as a measurement of drought tolerance. Having a lower (more negative) π_{TLP} indicates greater drought tolerance, as it means the plant is able to resist wilting even at a low Ψ_{leaf} (Turner

and Jones 1980; Morgan 1984; Bartlett et al. 2012b). A low π_{TLP} is advantageous because when a plant wilts, the lack of turgor pressure prevents growth and causes the stomata to close, preventing carbon intake for photosynthesis. Additionally, π_{TLP} determines the soil water potential at which the plant can no longer recover from wilting, or the permanent wilting point (Bartlett et al. 2012b). Plants can decrease π_{TLP} when under drought stress by accumulating solutes such as ions, sugars, amino acids, organic acids, amines, and polyols; the amount of adjustment possible may vary among species due to differences in the metabolic cost of accumulating a particular type of solute (Morgan 1984; Nilsen and Orcutt 1996; Bartlett et al. 2014).

Alternatively, evidence of plant response to drought can be obtained through gene expression data, which precede ecophysiological changes (De Nadal et al. 2011; Jończyk et al. 2017), and can capture a greater diversity of responses beyond measured physiological traits. Specifically, gene expression can be assessed by measuring expression levels of all mRNA found in the tissue using RNA-seq (Finotello and Camillo 2014), which can give a fairly complete picture of which genes are expressed in a tissue. One way to identify gene expression response to abiotic stress is to compare the gene expression of individuals under controlled and experimentally stressed conditions and then identify genes that are differentially expressed (DE) between the two conditions. A variety of statistical methods have been developed for detecting DE genes (Soneson and Delorenzi 2013). Moreover, gene expression data can be analyzed by grouping co-expressed genes together into eigengenes or 'modules' as implemented in weighted gene co-expression analysis (WGCNA, Langfelder and Horvath 2008). This approach allows grouping of genes into putative functional categories which may be regulated in the same way, and provides a module

expression value that allows each module to be tested for upregulation or downregulation under different conditions. Such modules can also be assigned putative functions based on the functions of member genes, which may allow determination of which pathways are regulated under certain conditions or induced by certain signals.

Previous studies on both model and non-model plant species have identified many genes and pathways involved in drought response. Major drought response pathways identified from *Arabidopsis* are often divided into two groups, based on whether their expression levels rely on levels of abscisic acid (ABA): ABA-dependent and ABA-independent (Bray 2004; Shinozaki and Yamaguchi-Shinozaki 2007; Todaka et al. 2015). ABA is produced during drought stress and is involved in stomatal closure and regulation of the expression of genes related to drought response (Seki et al. 2007; Shinozaki and Yamaguchi-Shinozaki 2007). Studies on crop species have confirmed the importance of genes identified from model-species studies, as well as discovered additional genes and pathways (Shanker et al. 2014). Additionally, many studies on non-model species have identified candidate genes involved in drought response based on expression levels in control and water stressed plants, including *Populus* species (Street et al. 2006; Cohen et al. 2010), wild barley (Hübner et al. 2015), switchgrass (Meyer et al. 2014), fir (Behringer et al. 2015), pine and spruce (Yeaman et al. 2014), eucalyptus (Villar et al. 2011), and oaks (Gugger et al. 2016b; Steele 2017). Some studies have also used gene expression to study local adaptation to drought. For example, one *Arabidopsis* accession was found to upregulate a greater number of drought-responsive genes under water stress than other accessions, which may be due to strong upregulation of genes involved in signaling regulation (Des Marais et al. 2012). These previous

studies have shown that some drought responses are fundamental and shared among most seed plants, while some species- or population-specific responses may have evolved more recently to cope with a particular environment.

Differential expression studies can be useful in identifying stress response differences among taxa, these differences are frequently due to neutral variation (Tirosh and Barkai 2011). The generally strong selective pressure caused by drought means that many genes that change their expression levels in response to drought are likely to be involved in drought adaptation; however, it can be difficult to distinguish these genes from those with neutral variation in gene expression levels. Because of these non-adaptive responses, it can be useful to relate gene expression to other phenotypic traits, such as ecophysiological traits that have been previously identified as affecting fitness, in order to increase the confidence that a particular gene is involved in a functional trait, and therefore may be under selective pressure. This may be done by simply testing for correlations of the gene expression level and trait values (Tohge and Fernie 2012). This strategy has been used to study physiological stress responses to soil drying in switchgrass (Meyer et al. 2014) and *Arabidopsis* (Des Marais et al. 2012); carotenoid (Lee et al. 2012) and metabolite (Mounet et al. 2009; Osorio et al. 2011) content in tomato fruits; metabolites in *Arabidopsis* (Allen et al. 2010); and biomass in *Arabidopsis* (Sulpice et al. 2009). Similarly, some studies have used WGCNA to correlate physiological traits with module expression (rather than individual gene expression) in non-model species (e.g. Akman et al. 2015; Kenkel and Matz 2016). However, few studies in plants have attempted to relate expression levels and physiological measurements in order to provide evidence of gene function, and there is still much that is unknown about which particular

genes contribute to the plant's response (Pinheiro and Chaves 2011). One barrier to this strategy is that measurement of ecophysiological traits may involve destructive sampling, so individuals cannot be sampled both before and after a treatment in order to avoid confounding the response to treatment and response to damage.

When an experiment includes multiple treatments and individuals from multiple populations, an ANOVA framework can be used to identify species-wide responses, populations differences in response, and phenotypic plasticity. Using this framework, traits (including both gene expression and ecophysiology) can be assigned to three functional categories based on how they vary among conditions and populations: they may have environmental, genotypic, and genotype × environmental effects. A genotype (G) effect, meaning that genotypes are different, but do not respond to the environmental variable being measured, is due to differences among populations (which may be adaptive or neutral) that are not involved in the response. An environment (E) effect means that the trait varies among treatments, but the change is similar for all populations. This can be indicative of species-wide phenotypic plasticity. A genotype by environment (G×E) effect, meaning that different populations respond to the treatment in different ways, indicates a phenotypically plastic trait with a genetic basis. This type of effect may indicate that genotypes have evolved different responses to the environment due to local adaptation (Des Marais et al. 2013; Lasky et al. 2014), and traits with a G × E effect can be considered candidate locally adapted genes. This may occur due to differences in local climate among populations, particularly in the level of abiotic stress that individuals experience, resulting in selection pressure for different types of responses or different levels of plasticity at different

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sites. However, these population differences may also be due to neutral variation.

Valley oaks (*Quercus lobata*), a California endemic tree species with a wide climatic range, are an ideal system for studying tree response to environmental heterogeneity in water availability. In contrast to many tree species, valley oak populations have had consistent ranges over the past several hundred thousand years, so genetic differentiation is more likely to be the result of adaptive processes than demographic changes resulting from changes in glaciation (Gugger et al. 2013). Previous studies have provided evidence that valley oak populations are locally adapted, as would be expected from the climatic variation within their range (Gugger et al. 2013, 2016a; Sork et al. 2016). This means that differences between populations that persist despite gene flow are more likely to be a result of strong selection pressure by the environment (Kawecki and Ebert 2004), as opposed to genetic drift.

The overall goal of this study is to test the hypothesis that California populations of *Quercus lobata* respond differently to water stress as a result of local adaptation. We will assess the nature of ecophysiological and gene expression response to water stress of valley oak seedling populations from diverse climates throughout California by measuring the extent to which they show a similar species-wide response to soil drying, exhibiting only phenotypic plasticity; or respond differently, reflecting local adaptation. To test this central hypothesis, we compared the ecophysiological traits and gene expression of seedlings originating from six different populations throughout California under well-watered and soil drying conditions. Species-wide responses are defined here as physiological responses that respond to treatment in the same way for all populations, or genes that are differentially expressed in response to treatment in all populations.

Additionally, gene expression was related to drought-responsive ecophysiological traits by comparing individual gene expression and module expression to π_{TLP} and Ψ_{leaf} measurements for each individual. The specific objectives of this study are to 1) measure the physiological response to soil drying by measuring turgor loss point and leaf water potential in young seedlings derived from different localities, 2) determine their change in gene expression in leaf tissue, 3) identify genes that are important in water stress response through differential expression analyses and 4) assess the relationship of their expression with ecophysiological traits. Findings from this study will clarify the extent to which a widespread tree species responds locally to water stress, providing information about how this species will respond to future climate changes.

Methods

Sampling and Experiment Design

Acorns for the experiment were collected in fall 2012 from six sites throughout California (Figure 1): Malibu Creek State Park (MC), Fort Tejon State Historic Park (FT), Fort Hunter Liggett (FH), Centerville (CV), Platina (PL), and Redding (RD), as part of a larger common garden experiment. The locations have contrasting climates, with varying temperature, precipitation, and seasonality (Figure 1). A total of 93 seedlings were included, with 15-16 individuals from a single location (Table 1). Preparation and growth of seedlings is fully described in Delfino Mix et al. (2015). Briefly, acorns were stored at 1.1º C then sterilized with a 10% bleach solution to kill mold. Acorns were then grown together in a greenhouse at the U.S. Department of Agriculture Forest Service, Institute of Forest Genetics, Placerville, California. After about a year,

seedlings were moved to the University of California, Los Angeles campus, where experiments took place in a greenhouse in September 2013.

Seedlings were placed in a well-watered control group or a soil drying treatment group, with 3 or 4 individuals from one population in each treatment (specific sample sizes are given in Table 1). All seedlings in the water stress group were subjected to a drought-hardening period in which they were not watered for a period, then re-watered in order to allow recovery (Figure 2). This design was intended to mimic natural conditions in which seedlings are subject to intermittent periods of drought over a long period of time, which facilitates acclimation by seedlings (Vilagrosa et al. 2003; Villar-Salvador et al. 2004). After drought-hardening, water was withheld again.

Ecophysiological measurements and analysis

Leaf tissue for RNA-seq was collected 10 days after the second dry-down treatment began. This length of time was chosen because it was expected that seedlings would be in an early stage of water stress response. A previous study sampled seedlings after 15 days of soil-drying and found that many of the differentially expressed genes were related to death and senescence (Gugger et al. 2016b), so a shorter period was used here to avoid measuring the effects of severe stress. Ecophysiology measurements were sampled both at 10 days and at 20 days. The 10-day measurements were collected for all seedlings for which RNA-seq tissue was collected so that the relationship between gene expression and ecophysiology could be tested, and as evidence of plant response for use in interpreting RNA-seq results. Because there may be a lag between a change in

gene expression and a change in ecophysiology, ecophysiology measurements were also taken after another 20 days to determine how any differences in gene expression affected seedlings later in the stress response. Seedlings were only sampled once to avoid confounding the effect of destructive sampling and treatment on seedlings.

The following measurements were collected for each individual after 10 or 20 days of treatment: largest leaf length, width, and thickness; leaf thickness, area, and dry mass (average of two leaves); leaf water potential and turgor loss point (average of two measurements); and soil mass and soil water potential. Turgor loss point and leaf water potential were measured using an osmometer (this procedure for measuring turgor loss point is described in Bartlett et al. (2012a). To determine which measurements had a G, E, or G×E pattern, an ANOVA was performed in R using the 'aov' function with a 'trait ~ population × treatment' model. For significant results, pairwise t-tests were done among all groups.

RNA extraction and sequencing

After collection, leaves were immediately frozen in liquid nitrogen, then stored in a freezer at -80º C. RNA was extracted in spring and summer of 2016. Polyphenolics and polysaccharides were removed from leaves using a lithium chloride/urea-based pre-wash protocol originally developed for conifers (http://openwetware.org/wiki/Conifer_RNA_prep). Whole RNA was then extracted using the Qiagen RNeasy Plant Mini Kit protocol. The complete protocol is described in detail at: http://www.openwetware.org/wiki/Sork_Lab:Protocols#RNA_Extraction_for_Oak. Briefly, about 50 mg of frozen leaf tissue was ground in grinding tubes, and placed in an RNA

extraction buffer consisting of (per sample) 0.675 mL LiCl, 0.864 g urea, 0.288 mL 11% PVP K-60

solution, and 0.018 mL dithiothreitol. Samples were kept at 4 °C overnight. The next morning

(after about 15-18 hours), the Qiagen protocol was followed, including the optional step for

addition of DNase. RNA quality was checked with a Nanodrop, and samples with a low 260/280

(<1.5) or 260/230 (<1.4) ratio were purified using Agilent AMPure beads with a 70% ratio of

beads to RNA sample. Final RNA quality was checked using an Agilent TapeStation 2200.

Library preparation on the extracted RNA samples was done in three batches using an

Illumina NeoPrep and a TruSeq Stranded mRNA kit (v1). Library quality was checked again on

the TapeStation. Samples were diluted to 10 nM in a solution of 0.1% tween in Qiagen EB buffer,

based on molar concentrations calculated from the cDNA peak and concentration given by the

TapeStation. Samples were pooled, and AMPure bead purification was done on pooled libraries

using a 1:1 ratio of beads and sample in order to remove primer-dimers. Libraries were sequenced

using single-end, 50 bp sequencing on Illumina 4000 across four lanes (10 or 11 samples per

lane). Samples were assigned to lanes using a balanced design taking into account the home site,

maternal family, treatment, and library preparation batch of the sample. A total of 42 individuals

were sequenced, 22 from the control treatment and 20 from the soil drying treatment.

Samples were demultiplexed allowing one base mismatch in the barcode sequence. Reads

were trimmed using Cutadapt version 1.12 (Martin 2011) to trim adapters regions with a quality

score <27, and reads <20 bp long were removed. Reads were aligned to the *Q. lobata*

transcriptome (Cokus et al. 2015) using Bowtie2 end-to-end alignment with default 'sensitive'

parameters (Langmead and Salzberg 2012). Potential exclusion amplification (ExAmp) duplicates

were removed using a custom script that looked for identical sequences within a radius of 2500 pixels, and kept only the sequence with the highest quality score.

Read counts for a given mRNA feature (hereafter 'gene') were filtered to remove genes with 10 or fewer reads in 30 or fewer samples. Filtered read counts were transformed to log2-counts per million (logCPM), a continuous measurement of expression, using the 'voom' function in the R package limma (Ritchie et al. 2015). Technical effects of sequencing lane and library prep batch were regressed out by taking the residuals of a linear model of 'expression ~ lane + library preparation batch'. One sample (069-15) was a strong outlier based on clustering and PCA plots, so it was removed from further analyses.

Analysis of gene expression

Differential expression analysis was done using the 'eBayes' function in limma for the following linear models: 1) expression ~ treatment, to test for differentially expressed (DE) genes between the control and soil drying treatment for all individuals; 2) expression ~ site + treatment, to test for DE genes between the control and soil drying treatment while controlling for effects of home site; and 3) expression ~ site × treatment, to test for DE genes which respond to treatment differently among seedlings from different home sites. P-values were corrected for multiple testing using the Benjami-Hochberg procedure (false discovery rate = 0.05) (Benjamini and Hochberg 1995). The original alignment and duplicate-removed alignment were compared to each other to determine the effect of removing ExAmp duplicates, and there was little difference in the results; the p-values for the site × treatment model were strongly correlated with each other, with an R^2 of

0.965. Slightly fewer genes were found to be significantly DE in the duplicate-removed alignment. The duplicate-removed version was used for more conservative results. *Arabidopsis* orthologs and Pfam categories were identified for each gene using the annotated *Q. lobata* transcriptome (Cokus et al. 2015). Gene ontology enrichment testing was done for each group of significant DE genes using the R package GOseq, which accounts for bias in gene length (Young et al. 2010). Only genes that were annotated with TAIR orthologs and a predicted mRNA length were used in the analysis. The default Wallenius distribution method was used to approximate the null distribution and calculate p-values, as it is less computationally intensive.

Genes were sorted into "modules," groups of genes that are expressed similarly, using weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath 2008), an R package. Two individuals were identified as outliers and were removed. The 'blockwiseModules' function was used to assign modules in one block with a soft threshold power of 12 and the following parameters: power = 12, TOMType = "unsigned", minModuleSize = 30, reassignThreshold = 0, mergeCutHeight = 0.25, pamRespectsDendro = FALSE. Gene ontology enrichment testing was done using GOseq to identify possible functions for each module. A principle components analysis (PCA) of all treatment-correlated gene modules was used to group individuals and sites by their water stress response.

Relationship of ecophysiology and gene expression

Maximal information coefficients (MIC) were calculated using the minerva package in R (Albanese et al. 2013) to test for relationships between gene expression and π_{TLP} and Ψ_{leaf}. This test

allows both linear and non-linear relationships to be identified, as gene expression and physiological traits may have a non-linear relationship (Meyer et al. 2014). Tests were done only on the seedlings in the water stress treatment, in order to identify genes associated with individual variation in water stress response rather than genes that were up- or downregulated in the treatment compared to the control. For each expression-trait test, a null distribution of MIC values was created using 5000 bootstrapped MIC tests with randomly associated data points, and p-values were calculated based on the percentile of the actual MIC value in the upper part of the null distribution. Because using this method limits the resolution of lower p-values, they were not corrected for multiple testing, but values <0.001 were considered statistically significant; out of the 23683 tests, it would be expected that 23.6 of them would have p<0.001 by chance.

Pearson correlations were performed between WGCNA module expression and trait values for ecophysiological traits, as well as the presence or absence of water stress treatment, in order to test for relationships between module expression and trait variation among individuals.

Results

Ecophysiology

Among the traits testing for population differences, height, largest leaf thickness, and average leaf thickness were significantly different among individuals from different sites in the 10-day treatment (Table 2, Figure 3), after multiple testing correction (p<0.05). Among the treatment-responsive traits, π_{TLP} was significantly different between the control and treatment seedlings after 10 days, and both π_{TLP} and Ψ_{leaf} were significantly different between treatments after 20 days

(Tables 2,3). Additionally, soil mass and soil water potential measurements were different across treatments, confirming that soil drying reduced water content in pots (Figure 4). The 20-day control treatment had decreased soil mass compared to the 10-day control treatment, indicating that control plants could have suffered slight water stress, but mass was still significantly higher than that of the soil drying treatment at the same time points. No traits showed a significant site × treatment interaction.

Average π_{TLP} after 10 days of water stress treatment was 0.22 MPa lower than the control at the same time, and 0.70 MPa lower after 20 days (compared to the control at 10 days, which is more likely to be the most representative of the unstressed condition due to potentially lower water availability in the 20-day control compared the 10-day control). Similarly, Ψ_{leaf} was 0.27 MPa lower in the water stress treatment after 10 days compared to the control, and 1.74 MPa lower after 20 days compared to the 10-day control (Figure 4). Measurements of π_{TLP} and Ψ_{leaf} for each individual seedling are shown in Figures S2 and S3.

Gene expression

After all filtering steps, a total of 23683 *Quercus lobata* transcripts (mRNA features) were identified. The average number of reads per gene per sample (mean across samples of mean gene count for each sample) was 438 reads with a standard deviation of 217. The standard deviation of the average read count per gene was 1036.

Differential expression analysis

Volcano plots showing log fold expression change and p-values for all models are shown in Figure 5. No significant genes were found for the expression ~ treatment model, indicating no genes were differentially expressed in different treatments without accounting for the home site of seedlings. For the expression ~ site + treatment model, 1274 genes were differentially expressed in the soil drying treatment (588 upregulated, 686 downregulated). These included two treatment-upregulated heat shock proteins in the top ten DE genes. Gene ontology enrichment analysis resulted in 12 significantly enriched GO categories for this model; all of which were upregulated on average in the water stress treatment (Table 4), including response to heat, protein folding, and abscisic acid metabolic process.

For the site × treatment model, a total of 679 genes were differentially expressed between treatments for different sites. Seedlings from four sites had multiple genes that were DE between treatments (listed in order of number of genes): MC, CV, RD, and FH. FT and PL did not have any significantly DE genes. Most genes were DE for only one site, but a few were DE in two sites; no genes were shared among more than two sites (Figure 6). The group of DE genes for FH and RD did not have any significantly enriched GO categories, likely due to the low number of annotated DE genes. However, FH seedlings had a late embryogenesis abundant (LEA) protein upregulated under water stress, which have been found to be associated with drought in other species (Chaves et al. 2003; Bray 2004). RD seedlings had several ribosomal proteins upregulated under water stress, as well as ZINC INDUCED FACILITATOR-LIKE 1 protein, which has been annotated as regulating stomatal closure. The DE genes for MC were significantly enriched for 33

GO categories (Table 5), including GO terms related to oxidoreductase activity, catalytic activity, and metabolic processes. For all of these categories, the average expression of genes from a category and averaged across a site/treatment combination was higher under the water stress treatment for most sites, but was particularly increased for MC (Figure 7A). CV had five significantly enriched terms (Table 5), all related to chloroplasts/plastids. For each of these GO categories, expression under the control treatment for CV was very low, and expression was increased under water stress treatment (Figure 7B).

Relationship of ecophysiology and gene expression

WGCNA identified 21 modules of coexpressed genes. Module expression, calculated as the first principle component of the module, was used to represent the overall expression of the module, although individual genes may not follow the same pattern (Langfelder and Horvath 2008). Correlations of module expression with traits are shown in Figure 8 and information for each module is shown in Table 6. Four modules were significantly positively correlated with the water stress treatment (upregulated), and six modules were negatively correlated with water stress treatment (downregulated). Additionally, one module (brown) was negatively correlated with π_{TLP} (i.e., seedlings with low π_{TLP} had high expression of the module), but not correlated with treatment, unlike other π_{TLP}-correlated modules. Representative examples of GO terms significantly enriched among the genes for each module are shown in Table 6. Module expression varied among sites for some modules; for example, the cyan module was positively correlated with water stress treatment, but only three sites had an increase in average expression, while the

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other three had similar average expression in both treatments. G×E plots of module expression for selected treatment-responsive modules with clear differences among populations and potential functions identified by GO enrichment are shown in Figure 9A-E, and plots for all modules are in Figure S1. A PCA of module expression for treatment-responsive modules (those with significant correlation of expression with treatment) is shown in Figure 9F. This shows CV, MC, and RD seedlings clustering together, PL and FH together, and FT furthest from other populations.

MIC tests identified genes with a strong relationship between their expression and the π_{TLP} and Ψ_{leaf} of each individual seedling under water stress treatment. Overall, Ψ_{leaf}-expression relationships had higher MIC values and lower p-values, with 139 genes significant at a $p<0.001$ level. Tests for π_{TLP} identified only 25 genes with $p<0.001$, close to the 23.7 expected by random chance. The gene modules from WGCNA were identified for the significant genes (Table 6). The proportion of genes related to Ψ_{leaf} included in the green, midnightblue, red, and salmon modules, which were positively correlated with water stress treatment, as well as the brown module, which was correlated with π_{TLP}, was higher than the proportion total proportion of genes in those modules. The relationship between gene expression and Ψ_{leaf} for all genes with $p<0.001$ is shown in Figure 10, illustrating that many of these relationships appear to be nonlinear.

Discussion

Species-wide responses

Ecophysiology

The drought-responsive traits (π_{TLP} and Ψ_{leaf}) showed significant differences among treatments and time points but not among populations, indicating that valley oaks have a consistent physiological response to water stress across populations. Reduction in π_{TLP} is a common plant drought response to avoid wilting and maintain normal functions, and its value under well-watered conditions and the degree of change vary among species. The average π_{TLP} after 20 days of water stress was 0.70 MPa lower compared to the unstressed 10-day control, which was a greater reduction than the average of 0.44 MPa reported in a meta-analysis by Bartlett et al. (2014), indicating that valley oaks seedlings have more plasticity in π_{TLP} on average than 317 other species that were included in the analysis. This degree of adjustment is most similar to the average difference for species from medium/dry temperate and semidesert climates reported by the study (-0.61 and -0.55 MPa, respectively).

Differential gene expression

Differential expression analysis indicated that there was no universal gene expression response to treatment for these seedlings. When controlling for the effects of the home site, many DE genes were identified which had functions expected for drought response, possibly indicating that some responses are shared among multiple sites. Interestingly, genes with the GO term "response to karrikins" were significantly enriched in this group of genes. Karrikins were

originally discovered from burned plant materials and are related to germination, but there has been some evidence that karrikins are also involved in stress responses, and may play a role similar to that of ABA (Li and Tran 2015).

A previous study in valley oak found that 52% of contigs responded to a water stress treatment (Gugger et al. 2016b), which is a much stronger response than found here. This may be a result of the differences in treatment between the studies; the previous one was longer (15 days instead of 10) and did not include an pre-treatment with recovery. In this study, Ψ_{leaf} measurements indicated that stress was not very severe after 10 days of treatment (Figure 4), so the seedlings in the previous study may have been under more severe stress, resulting in a large number of genes being induced (many of which were related to stress response and death). Together these results suggest that valley oak populations respond differently to water stress at earlier stages, potentially due to local adaptation; but when stress is severe, they may have more shared responses than population-specific responses.

Candidate genes identified by relationship with leaf water potential

We did not find universal water stress genes using differential expression analysis, but the measurement of ecophysiological traits provides a different source of data to investigate their relationship with gene expression. Because water stress responsive traits were variable but not significantly different among populations at 10 days, each seedling can be considered a replicate at a slightly different level of stress (likely due to slight variation in experimental conditions). When gene expression levels vary with ecophysiology, that gene may either be induced by that trait or

involved in causing the phenotype. Either of these possibilities indicate that the function of the gene may be related to the water stress response. Many genes had expression that was significantly associated with Ψ_{leaf} during water stress. Because Ψ_{leaf} can be considered a measurement of water stress, a gene with increased expression under low Ψ_{leaf} may be a drought-responsive gene controlled by osmotic status or turgor pressure in leaves (Chaves et al. 2003; Reddy et al. 2004; Osakabe et al. 2014).

The relationship of expression with Ψ_{leaf} for many of these genes was nonlinear, similar to the pattern found by Meyer et al. (2014). If individuals with different values of Ψ_{leaf} can be considered to be at different stages of osmotic stress, the stage at which the decrease in Ψ_{leaf} begins to alter gene expression appears to be around -1 to -1.5 MPa (Figure 11). Around this point, gene expression either decreased to reach constant levels of expression (for genes downregulated under low Ψ_{leaf}), or increased from previously-constant expression levels (genes upregulated under low Ψ_{leaf}), suggesting that cells begin to detect osmotic stress or turgor loss and alter their gene expression around this value of Ψ_{leaf}. However, this result could also be explained by uneven distribution of Ψ_{leaf} values among individuals, so further studies are needed to determine whether this is a general trend. Many of the genes with increased expression under low Ψ_{leaf} belonged to the treatment-upregulated green, salmon, and midnightblue modules, as well as the π_{TLP}-related brown module, so Ψ_{leaf} may be acting as part of a pathway signaling water stress for these modules (see Table 6 for total numbers of genes related to Ψ_{leaf} in each module).

Population-specific responses

Differential gene expression

DE analysis indicated a strong G×E effect among populations, with the different levels of response among populations ranging from 321 DE genes to none (Figure 6). Having a large number of DE genes may be indicative of either an adaptive stress response in more tolerant genotypes, or of greater stress in more sensitive genotypes (DeBiasse and Kelly 2016). The correct interpretation likely depends on variation in the level and duration of the stress as well as the species being studied, so the function of the DE genes is important for interpreting whether a response is adaptive or due to major stress. For example, Villar et al. (2011) found that a drought-tolerant genotype of eucalyptus trees had more upregulated genes than a sensitive genotype when unirrigated during the dry season, and that photosynthesis-related genes were downregulated while water stress response genes were upregulated under non-irrigated conditions in the tolerant genotype. Another study used a 60 day greenhouse drought treatment in clover, in which the sensitive genotype had more DE genes, but also upregulated more genes involved in cell death, heat response, and water stimulus, possibly signifying a greater degree of stress (Yates et al. 2014). Because the valley oak populations in this study did not significantly differ in ecophysiology, it is difficult to determine which were more tolerant; however, the functions of DE genes are indicative of water stress response rather than cell death/senescence, so they may be a result of drought adaptation. The number of DE genes does not show a clear pattern with climatic variables across all populations. However, MC and CV, the two most responsive populations, had the two highest home site climatic water deficit (CWD) measurements, which can be thought of as a

measurement of water demand (calculated as actual evapotranspiration minus potential evapotranspiration). This suggests that the responses of these seedlings may be a result of local adaptation to the higher drought stress at their home sites. RD, with a moderate number of DE genes, has the greatest amount of summer precipitation, but also has hot summers, so may experience drought conditions in periods between rainfall. Together these results suggest that some populations have more DE genes due to climatic conditions which result in local adaptation to survive California's combined summer heat and drought.

In addition to variation in the degree of plasticity, populations had functionally different responses to drought. MC seedlings had 321 DE genes, which were enriched for GO terms related to oxidoreductase activity (these genes were upregulated on average, Figure 7A). Oxidoreductase activity may be involved in protecting cells from damage by scavenging the reactive oxygen species (ROS) that accumulate under water stress, as photosynthesis and CO_2 fixation ceases and the electron transport chain becomes over-reduced (Chaves et al. 2003; Miller et al. 2010). Additionally, three upregulated genes were related to flavonoid biosynthesis, which can protect leaves against ROS damage by screening UV light and/or by directly acting as antioxidants and scavenging ROS (Gill and Tuteja 2010; Pollastri and Tattini 2011; Takahashi and Badger 2011). Together these results suggest that MC seedlings prioritize reducing ROS damage under water stress more than other seedlings, perhaps because their home site is more prone to a combination of drought and high light or heat stress which exacerbates the generation of ROS.

The GO terms enriched in significant DE genes for CV were cellular structure terms related to plastids, and the genes were on average upregulated under the water stress treatment; however, the primary reason for the strong increase was that control expression was much lower compared

to the other populations (Figure 7B). These genes may be involved in repairing damage to the chloroplasts from ROS by replacing structures. Alternatively, they may be involved in changes in carbohydrate metabolism which may occur during water stress (Pinheiro and Chaves 2011; Des Marais et al. 2012). One possibility is that they are involved in regulating π_{TLP}, which decreases the most (although not significantly) in the CV seedlings, primarily due to high values in control seedlings – the same pattern as seen in plastid gene expression (Figure S2). Starch is stored in the chloroplasts, and previous studies in oak species have determined that osmotic adjustment seems to occur by accumulation of glucose and fructose (Épron and Dreyer 1996; Gebre and Tschaplinski 2002) while starch and sucrose decreases (Épron and Dreyer 1996). Another piece of evidence that may support the hypothesis that these plastid-related genes could be involved in drought response is that the gene m01oak03811CC, identified as an ortholog to the *Arabidopsis* gene DSP4/SEX4, is upregulated under water stress in CV seedlings. This gene is potentially involved in the degradation of starch to sucrose in the chloroplasts (Kerk et al. 2006; Niittylä et al. 2006; Berrocal-Lobo et al. 2011), suggesting that it could be involved in an early step in the accumulation of these solutes in the cytoplasm, which causes π_{TLP} to decrease.

Seedlings from FH and RD had a small number of DE genes, and GO enrichment analysis did not identify any significant categories. However, some genes indicate water stress response in these seedlings. In FH seedlings, late embryogenesis abundant 7 (LEA7) was strongly upregulated under water stress (log fold change = 5.81). LEA proteins have been shown to be associated with drought tolerance and may involved in protecting the cell from damage due to dehydration (Bartels and Sunkar 2005; Shinozaki and Yamaguchi-Shinozaki 2007). In RD, five ribosomal-

related proteins were upregulated in response to water stress. The function of these genes is less clear, but they could potentially be involved in transcription of new proteins, either to replace damaged proteins or due to upregulation of proteins involved in water stress response.

Treatment-related gene modules

While several gene modules that correlated with treatment were identified, the change in module expression for each population often varied, with some populations changing module expression more strongly than others. A PCA of the module expression of treatment-responsive modules (those that correlated with treatment) for the water-stressed individuals shows that MC, CV, and RD seedlings cluster together, indicating similar responses of drought-responsive modules (Figure 9F). When comparing the expression of modules with apparent stress-related functions, a similar pattern emerges (summarized in Figure 10): MC and CV downregulate photosynthesis genes (magenta module), but less strongly than other populations, and strongly upregulate UV response genes (midnightblue module), upregulate translation genes (cyan module), and downregulate protein catabolism genes (red module). FH and PL downregulate photosynthesis genes and upregulate stress response genes (green module) more strongly, but have little change in translation, UV response, and protein catabolism genes. RD seedlings are intermediate between these two groups for several modules, and FT seedlings have little change in the expression of any modules. Although a change in the expression of genes related to these processes does not necessarily mean that the processes themselves are different (for example, genes may have multiple functions that GO terms do not reflect), this pattern does indicate

distinct differences in water stress response among populations which may be a result of local adaptation to their home climates.

The different patterns of gene expression may point to functional differences in drought response among populations. Drought responses may be categorized as drought avoidance or drought tolerance. Drought avoidance (or isohydry), in which the stomata are closed early to preserve water at the cost of reduced photosynthesis, increases the risk of death due to carbon starvation; while drought tolerance (anisohydry), in which the stomata are kept open for longer to continue photosynthesis, but leaf water potential drops, increases the risk of death due to hydraulic failure (Mcdowell et al. 2008; Allen et al. 2010). Moran et al. (2017) also hypothesized that anisohydric individuals would increase expression of protective molecules earlier in the drought response in order to minimize damage due to low water potentials. This could explain the pattern in FH and PL as a more anisohydric response, with strongly increased stress response genes (involving hormones such as ABA signaling to close stomata), decreased photosynthesis gene expression (repression of photosynthesis due to carbon starvation), and less need for protective molecules involved in protein folding and UV response (due to better preservation of normal leaf water potential). CV and MC seedlings instead have greater expression of photosynthesis genes, less expression of stress response genes, and increased protein folding/UV response genes indicating a more isohydric response. While the red (protein catabolism) module is less clearly related, possibly due to many member genes of unknown function, one member 'hub gene' (a highly connected gene, Langfelder and Horvath 2008) is negatively correlated with the module expression, possibly indicating that it negatively regulates genes in the module. This gene, m01oak02352SC, was identified as an ortholog of the *Arabidopsis* HDA1 gene, which

reduces guard cell sensitivity to ABA when upregulated (Song et al. 2005), and is upregulated under water stress in MC, CV and RD seedlings, potentially allowing them to keep the stomata open for a longer period of time.

One caveat to this explanation is that "protective molecules" may be predicted to increase in both isohydric and anisohydric individuals for different reasons. Low water potential (as in anisohydric plants) may require a plant to increase the number of protective molecules, but they may also increase due to stomatal closure (as in isohydric plants), which reduces transpirational cooling and increases leaf temperature, as well as causes an increase in ROS due to excess electrons in photosynthesis reactions. However, these differences among populations do suggest different pathways, and it is reasonable that the populations in drought-prone environments (MC and CV) would have evolved to be more anisohydric to maintain normal functions under drought.

Conclusions

Long-lived plant species such as trees may live through many fluctuations in climate, including periodic droughts. When periodic climate stresses vary throughout the range of a species, populations may be locally adapted to these stresses. This study provides evidence that valley oak populations are locally adapted in their response to drought, and adds to the evidence that local adaptation is particularly important in allowing plants to survive environmental changes throughout their lives. Valley oak populations appear to have both quantitatively and qualitatively different responses to water stress. Some populations have very little change in gene

expression between the treatments, while some had hundreds of genes change. This can be considered variation in the level of plasticity in response to water stress in valley oak populations, and suggests a selective advantage of greater plasticity in certain climates, which results in local adaptation. The differences in types of genes responding to treatment suggest that populations from different climates also have qualitatively different responses to drought stress. Although variation in the type of response is typically thought of as a trait that varies among species, these are not discrete characteristics and exist along a continuum; so these populations may exist in different positions along the continuum of drought tolerance/anisohydry to drought avoidance/isohydry.

This study has important implications for climate change, as extreme droughts are projected to become more frequent in California and other regions (Trenberth 2011). In order to survive climate change, species must either migrate to new regions with a suitable climate or adapt to the new climate (Aitken et al. 2008), but as anthropogenic climate change is proceeding more rapidly than natural changes in climate, it is uncertain whether species will be able to migrate or adapt quickly enough to survive. Assisted gene flow may allow maintenance of valley oak populations, as it would transport individuals or gametes between populations to climates where they may be better adapted to future conditions than the current population (Aitken and Whitlock 2013). However, this would require knowledge of the adaptations and range of climatic tolerance of each population. This study suggests that some valley oak populations are less capable of responding to drought stress and may be more at risk as the frequency and severity of droughts increases, while other populations may be better adapted to drought and will survive longer. These better-adapted

populations could be candidates for assisted gene flow to the populations which will be under greater stress in the future. The fact that the magnitude of response was not clearly related to precipitation levels of the home site underscores the importance of testing a population's response before determining which populations are more vulnerable and which could be candidates for strategies such as assisted migration.
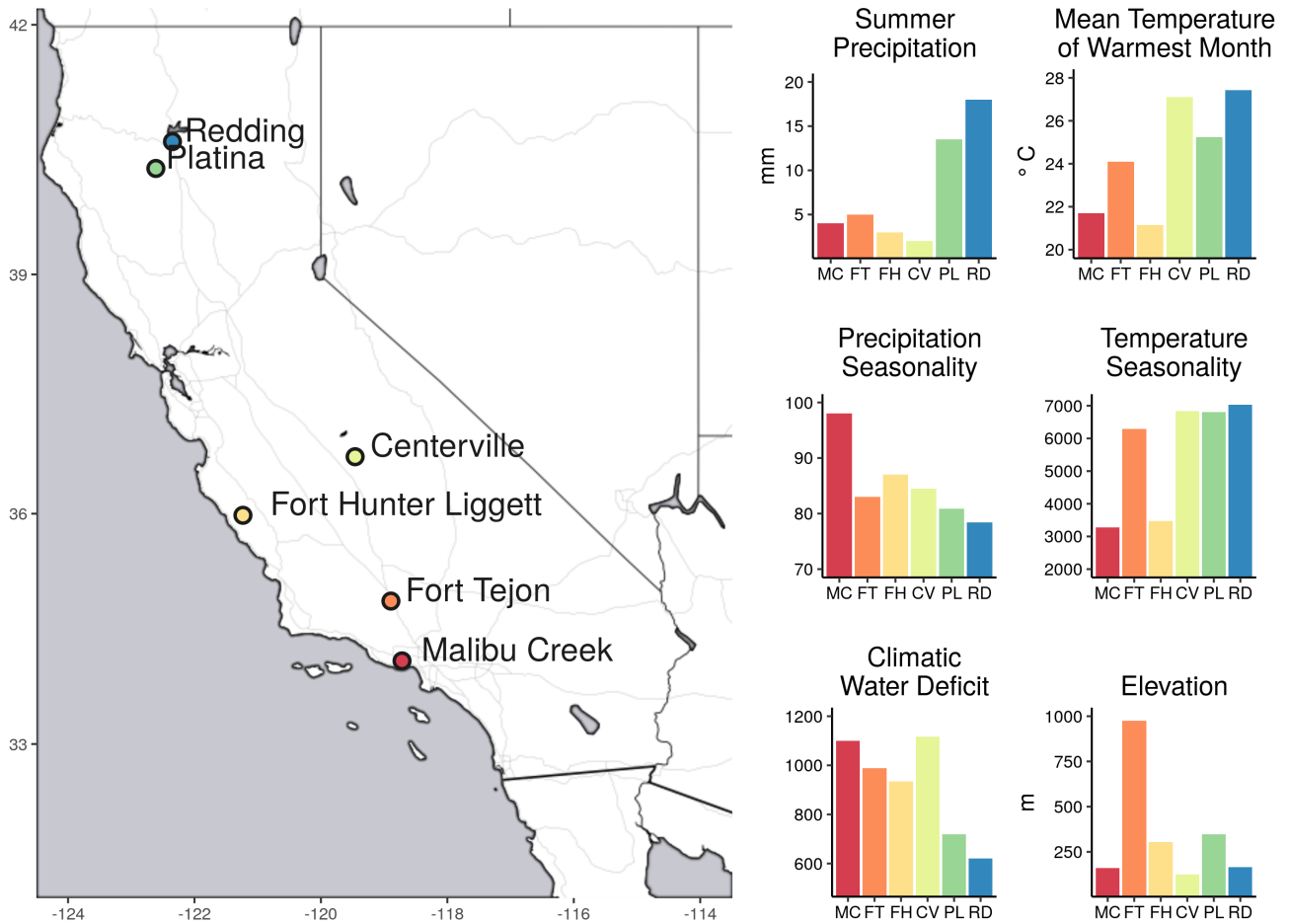
Figure 1. Map of sample sites of maternal parent trees of seedlings and climate variables for each site. Temperature seasonality and precipitation seasonality measurements are calculated from 30 years of data from 1971-2000, provided by the U.S. Geological Survey using Parameter-elevation Regression on Independent Slopes Model (PRISM) data (O'Donnell and Ignizio 2012). Climatic water deficit is for the years 1981-2010. Temperature seasonality is the temperature standard deviation * 100, precipitation seasonality is the coefficient of variation.
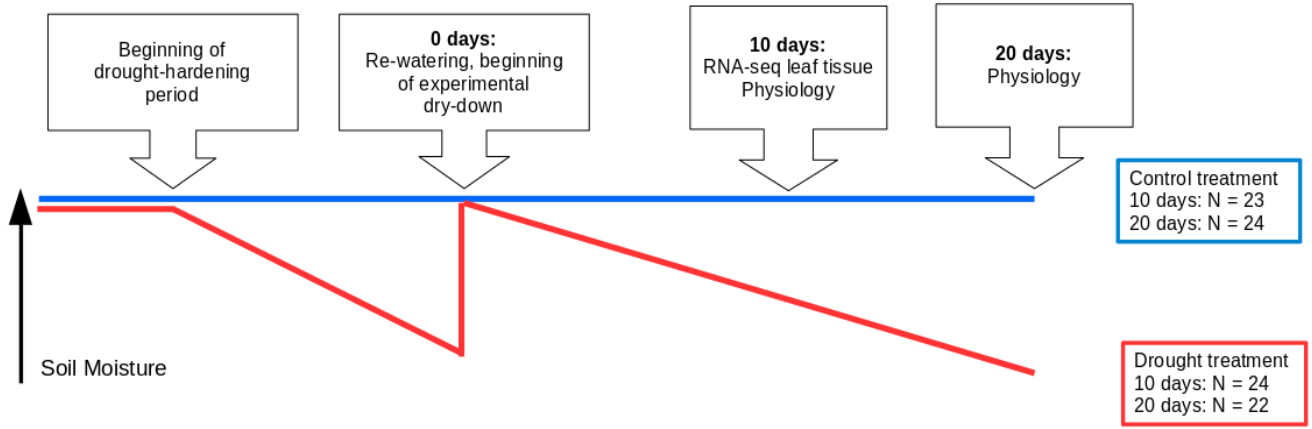
Figure 2. Diagram showing experimental design. After 10 days of either water stress or control treatment, leaf tissue was collected for RNA-seq, and ecophysiology measurements (described in Methods) were taken. After 20 days, only ecophysiology measurements were taken.
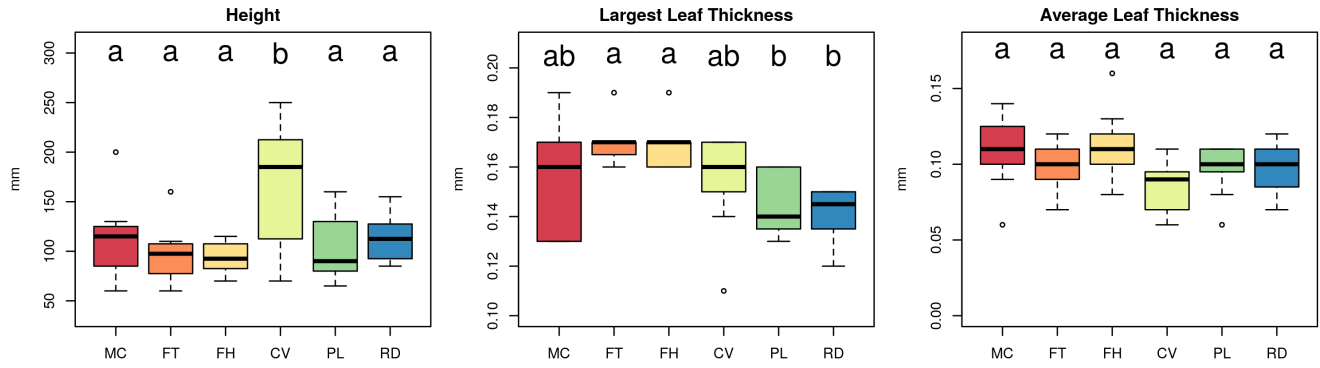
Figure 3. Differences among sites for traits with significantly more variation among populations than within, according to the ANOVA test. All individuals shown were from the 10-day measurements, with control and treatment seedlings pooled together. Letters above boxplots indicate groups that are significantly different from each other according to a post-hoc two-tailed t-test (p<0.05 after Benjamini-Hochberg correction).
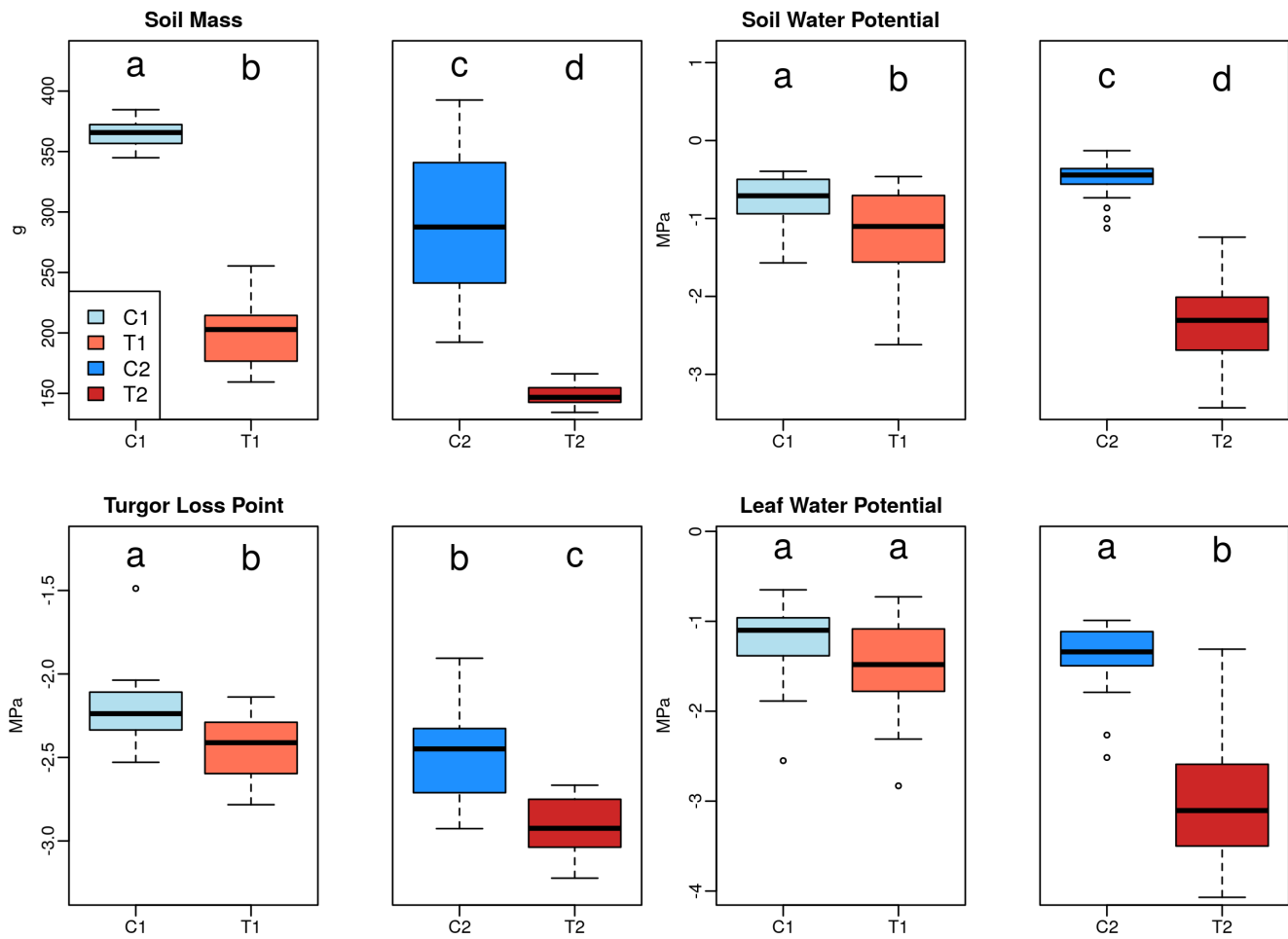
Figure 4. Differences among treatments for significantly different measurements. Letters above boxplots indicate groups that are significantly different from each other according to a two-tailed t-test (p<0.05 after Benjamini-Hochberg correction). C1 = 10-day control, T1 = 10-day treatment, C2 = 20-day control, T2 = 20-day treatment.
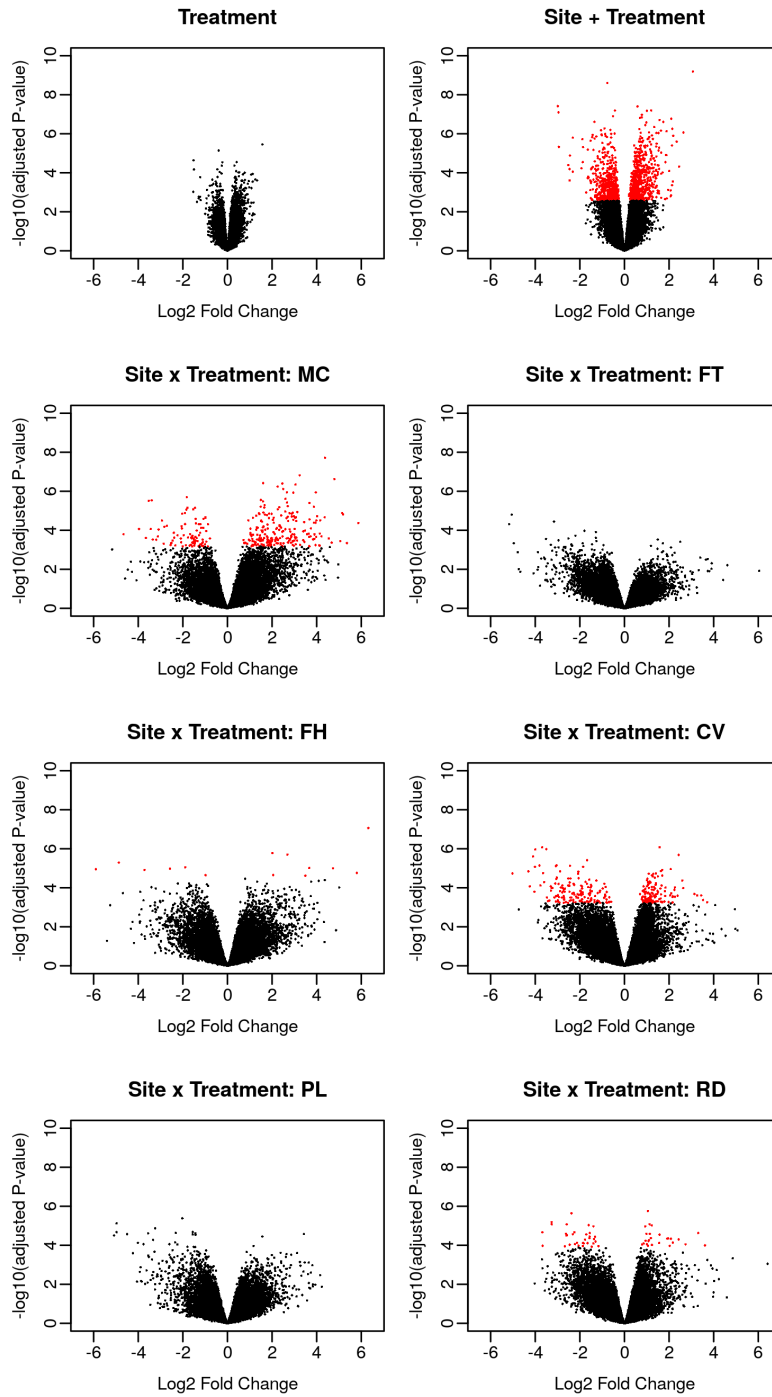
Figure 5. Volcano plots for each model tested in limma. P-values shown are those before correction for multiple tests; red points indicate genes that are significantly DE after p-value correction (FDR < 0.05).
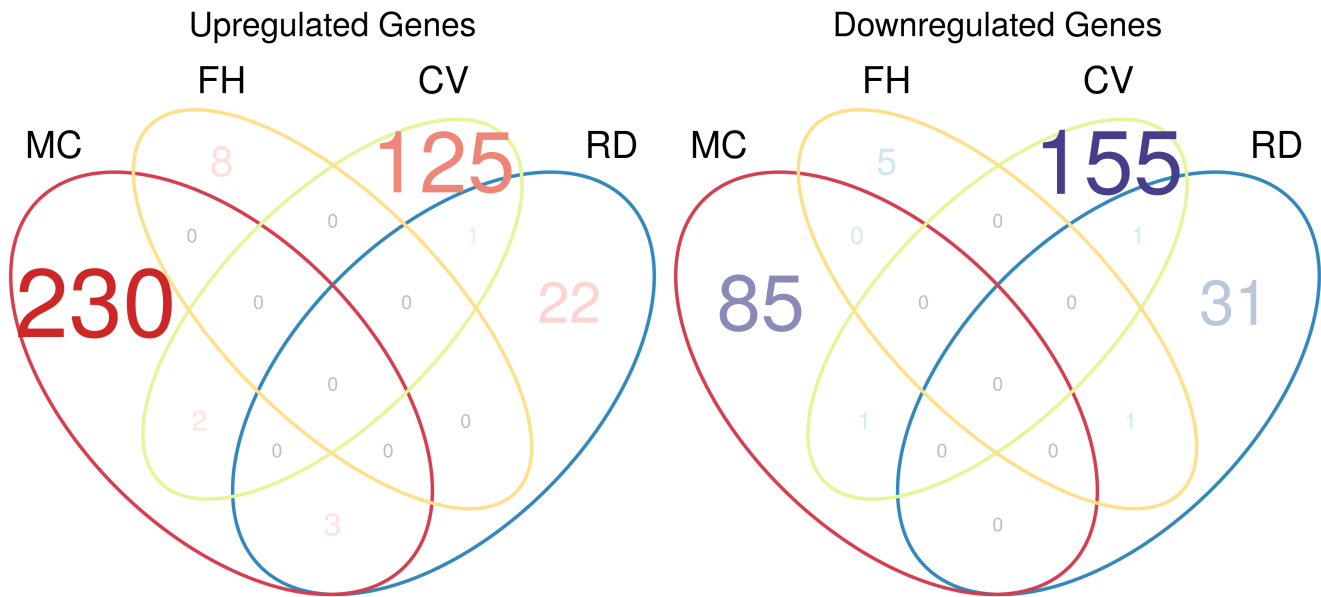
Figure 6. Venn diagrams showing the number of significant DE genes for each site using the site×treatment model. Genes are sorted by upregulation (left) or downregulation (right) under water stress treatment. Text size and colors are scaled to the number of genes. FT and PL were not included because they had no DE genes.
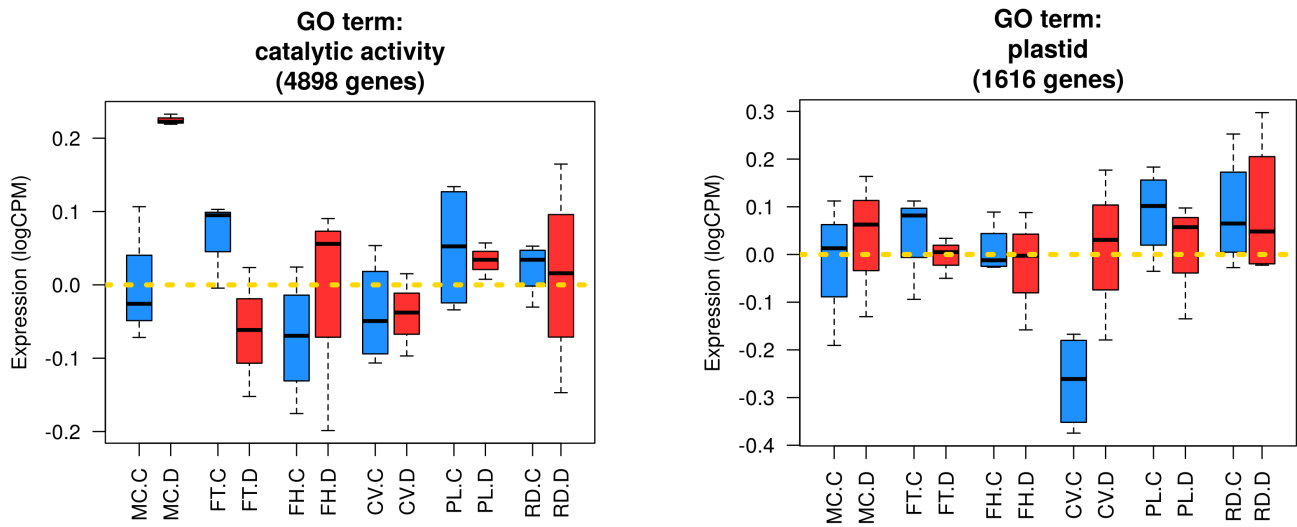
Figure 7. Plots of average gene expression of all genes in a GO category, averaged across all samples from a given site/treatment combination, for GO terms that are overrepresented in the significant site × treatment genes for A) MC seedlings, and B) CV seedlings. Significantly enriched GO terms for each site generally showed patterns similar to these plots. Blue ('C') boxplots are the control individuals, and red ('D') boxplots are the water stress treatment individuals.

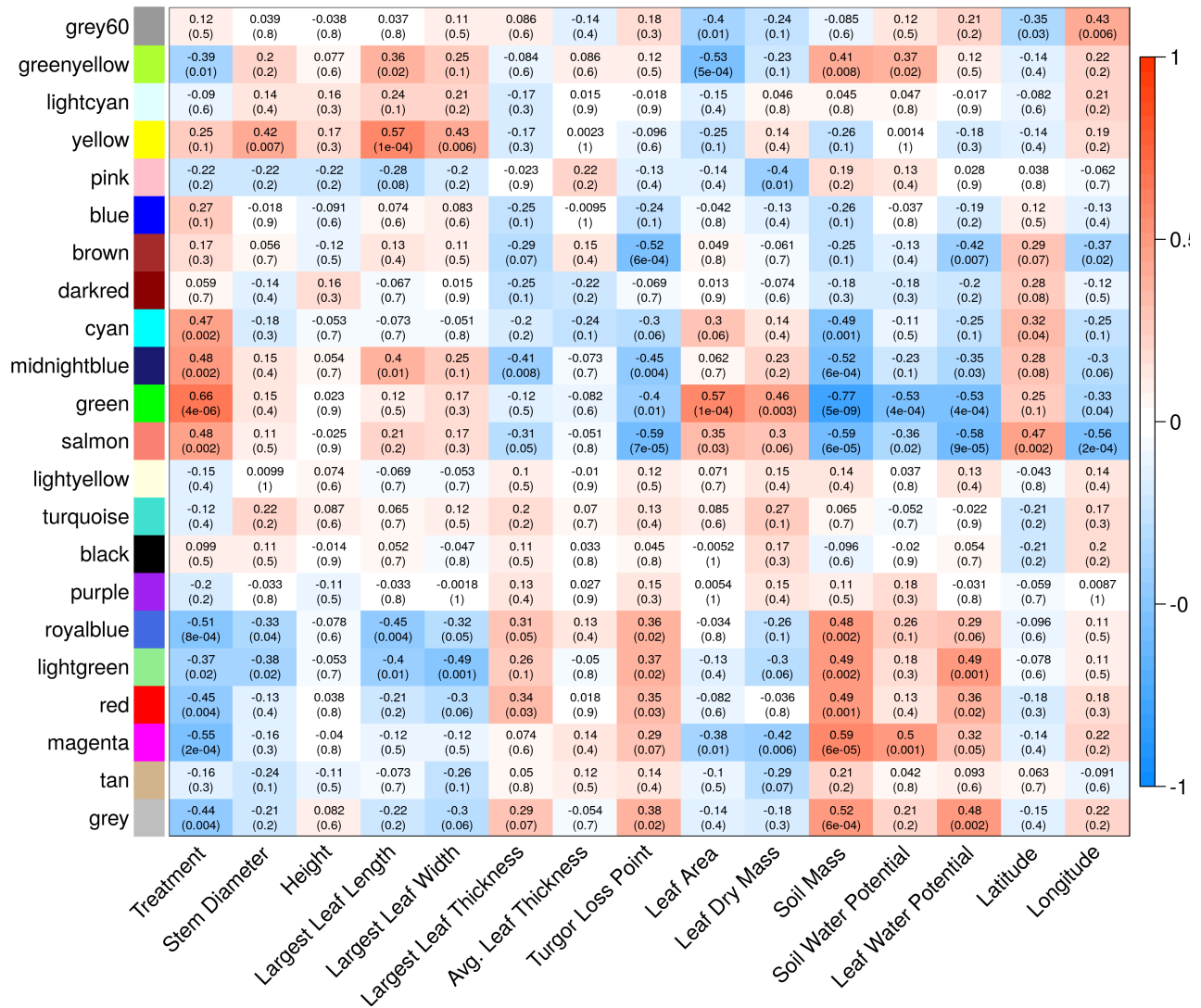| Module	Treatment	Stem Diameter	Height	Largest Leaf Length	Largest Leaf Width	Largest Leaf Thickness	Avg. Leaf Thickness	Turgor Loss Point	Leaf Area	Leaf Dry Mass	Soil Mass	Soil Water Potential	Leaf Water Potential	Latitude	Longitude
grey60	0.12 (0.5)	0.039 (0.8)	-0.038 (0.8)	0.037 (0.8)	0.11 (0.5)	0.086 (0.6)	-0.14 (0.4)	0.18 (0.3)	-0.4 (0.01)	-0.24 (0.1)	-0.085 (0.6)	0.12 (0.5)	0.21 (0.2)	-0.35 (0.03)	0.43 (0.006)
greenyellow	-0.39 (0.01)	0.2 (0.2)	0.077 (0.6)	0.36 (0.02)	0.25 (0.1)	-0.084 (0.6)	0.086 (0.6)	0.12 (0.5)	-0.53 (5e-04)	-0.23 (0.1)	0.41 (0.008)	0.37 (0.02)	0.12 (0.5)	-0.14 (0.4)	0.22 (0.2)
lightcyan	-0.09 (0.6)	0.14 (0.4)	0.16 (0.3)	0.24 (0.1)	0.21 (0.2)	-0.17 (0.3)	0.015 (0.9)	-0.018 (0.9)	-0.15 (0.4)	0.046 (0.8)	0.045 (0.8)	0.047 (0.8)	-0.017 (0.9)	-0.082 (0.6)	0.21 (0.2)
yellow	0.25 (0.1)	0.42 (0.007)	0.17 (0.3)	0.57 (1e-04)	0.43 (0.006)	-0.17 (0.3)	0.0023 (1)	-0.096 (0.6)	-0.25 (0.1)	0.14 (0.4)	-0.26 (0.1)	0.0014 (1)	-0.18 (0.3)	-0.14 (0.4)	0.19 (0.2)
pink	-0.22 (0.2)	-0.22 (0.2)	-0.22 (0.2)	-0.28 (0.08)	-0.2 (0.2)	-0.023 (0.9)	0.22 (0.2)	-0.13 (0.4)	-0.14 (0.4)	-0.4 (0.01)	0.19 (0.2)	0.13 (0.4)	0.028 (0.9)	0.038 (0.8)	-0.062 (0.7)
blue	0.27 (0.1)	-0.018 (0.9)	-0.091 (0.6)	0.074 (0.6)	0.083 (0.6)	-0.25 (0.1)	-0.0095 (1)	-0.24 (0.1)	-0.042 (0.8)	-0.13 (0.4)	-0.26 (0.1)	-0.037 (0.8)	-0.19 (0.2)	0.12 (0.5)	-0.13 (0.4)
brown	0.17 (0.3)	0.056 (0.7)	-0.12 (0.5)	0.13 (0.4)	0.11 (0.5)	-0.29 (0.07)	0.15 (0.4)	-0.52 (6e-04)	0.049 (0.8)	-0.061 (0.7)	-0.25 (0.1)	-0.13 (0.4)	-0.42 (0.007)	0.29 (0.07)	-0.37 (0.02)
darkred	0.059 (0.7)	-0.14 (0.4)	0.16 (0.3)	-0.067 (0.7)	0.015 (0.9)	-0.25 (0.1)	-0.22 (0.2)	-0.069 (0.7)	0.013 (0.9)	-0.074 (0.6)	-0.18 (0.3)	-0.18 (0.3)	-0.2 (0.2)	0.28 (0.08)	-0.12 (0.5)
cyan	0.47 (0.002)	-0.18 (0.3)	-0.053 (0.7)	-0.073 (0.7)	-0.051 (0.8)	-0.2 (0.2)	-0.24 (0.1)	-0.3 (0.06)	0.3 (0.06)	0.14 (0.4)	-0.49 (0.001)	-0.11 (0.5)	-0.25 (0.1)	0.32 (0.04)	-0.25 (0.1)
midnightblue	0.48 (0.002)	0.15 (0.4)	0.054 (0.7)	0.4 (0.01)	0.25 (0.1)	-0.41 (0.008)	-0.073 (0.7)	-0.45 (0.004)	0.062 (0.7)	0.23 (0.2)	-0.52 (6e-04)	-0.23 (0.1)	-0.35 (0.03)	0.28 (0.08)	-0.3 (0.06)
green	0.66 (4e-06)	0.15 (0.4)	0.023 (0.9)	0.12 (0.5)	0.17 (0.3)	-0.12 (0.5)	-0.082 (0.6)	-0.4 (0.01)	0.57 (1e-04)	0.46 (0.003)	-0.77 (5e-09)	-0.53 (4e-04)	-0.53 (4e-04)	0.25 (0.1)	-0.33 (0.04)
salmon	0.48 (0.002)	0.11 (0.5)	-0.025 (0.9)	0.21 (0.2)	0.17 (0.3)	-0.31 (0.05)	-0.051 (0.8)	-0.59 (7e-05)	0.35 (0.03)	0.3 (0.06)	-0.59 (6e-05)	-0.36 (0.02)	-0.58 (9e-05)	0.47 (0.002)	-0.56 (2e-04)
lightyellow	-0.15 (0.4)	0.0099 (1)	0.074 (0.6)	-0.069 (0.7)	-0.053 (0.7)	0.1 (0.5)	-0.01 (0.9)	0.12 (0.5)	0.071 (0.7)	0.15 (0.4)	0.14 (0.4)	0.037 (0.8)	0.13 (0.4)	-0.043 (0.8)	0.14 (0.4)
turquoise	-0.12 (0.4)	0.22 (0.2)	0.087 (0.6)	0.065 (0.7)	0.12 (0.5)	0.2 (0.2)	0.07 (0.7)	0.13 (0.4)	0.085 (0.6)	0.27 (0.1)	0.065 (0.7)	-0.052 (0.7)	-0.022 (0.9)	-0.21 (0.2)	0.17 (0.3)
black	0.099 (0.5)	0.11 (0.5)	-0.014 (0.9)	0.052 (0.7)	-0.047 (0.8)	0.11 (0.5)	0.033 (0.8)	0.045 (0.8)	-0.0052 (1)	0.17 (0.3)	-0.096 (0.6)	-0.02 (0.9)	0.054 (0.7)	-0.21 (0.2)	0.2 (0.2)
purple	-0.2 (0.2)	-0.033 (0.8)	-0.11 (0.5)	-0.033 (0.8)	-0.0018 (1)	0.13 (0.4)	0.027 (0.9)	0.15 (0.3)	0.0054 (1)	0.15 (0.4)	0.11 (0.5)	0.18 (0.3)	-0.031 (0.8)	-0.059 (0.7)	0.0087 (1)
royalblue	-0.51 (8e-04)	-0.33 (0.04)	-0.078 (0.6)	-0.45 (0.004)	-0.32 (0.05)	0.31 (0.05)	0.13 (0.4)	0.36 (0.02)	-0.034 (0.8)	-0.26 (0.1)	0.48 (0.002)	0.26 (0.1)	0.29 (0.06)	-0.096 (0.6)	0.11 (0.5)
lightgreen	-0.37 (0.02)	-0.38 (0.02)	-0.053 (0.7)	-0.4 (0.01)	-0.49 (0.001)	0.26 (0.1)	-0.05 (0.8)	0.37 (0.02)	-0.13 (0.4)	-0.3 (0.06)	0.49 (0.002)	0.18 (0.3)	0.49 (0.001)	-0.078 (0.6)	0.11 (0.5)
red	-0.45 (0.004)	-0.13 (0.4)	0.038 (0.8)	-0.21 (0.2)	-0.3 (0.06)	0.34 (0.03)	0.018 (0.9)	0.35 (0.03)	-0.082 (0.6)	-0.036 (0.8)	0.49 (0.001)	0.13 (0.4)	0.36 (0.02)	-0.18 (0.3)	0.18 (0.3)
magenta	-0.55 (2e-04)	-0.16 (0.3)	-0.04 (0.8)	-0.12 (0.5)	-0.12 (0.5)	0.074 (0.6)	0.14 (0.4)	0.29 (0.07)	-0.38 (0.01)	-0.42 (0.006)	0.59 (6e-05)	0.5 (0.001)	0.32 (0.05)	-0.14 (0.4)	0.22 (0.2)
tan	-0.16 (0.3)	-0.24 (0.1)	-0.11 (0.5)	-0.073 (0.7)	-0.26 (0.1)	0.05 (0.8)	0.12 (0.5)	0.14 (0.4)	-0.1 (0.5)	-0.29 (0.07)	0.21 (0.2)	0.042 (0.8)	0.093 (0.6)	0.063 (0.7)	-0.091 (0.6)
grey	-0.44 (0.004)	-0.21 (0.2)	0.082 (0.6)	-0.22 (0.2)	-0.3 (0.06)	0.29 (0.07)	-0.054 (0.7)	0.38 (0.02)	-0.14 (0.4)	-0.18 (0.3)	0.52 (6e-04)	0.21 (0.2)	0.48 (0.002)	-0.15 (0.4)	0.22 (0.2)

Figure 8. Correlations between module expression and traits. Colors on left are the modules, labeled with color names. The shade and top number in each cell indicates the correlation coefficient of module expression with the trait, with the p-value in parentheses.
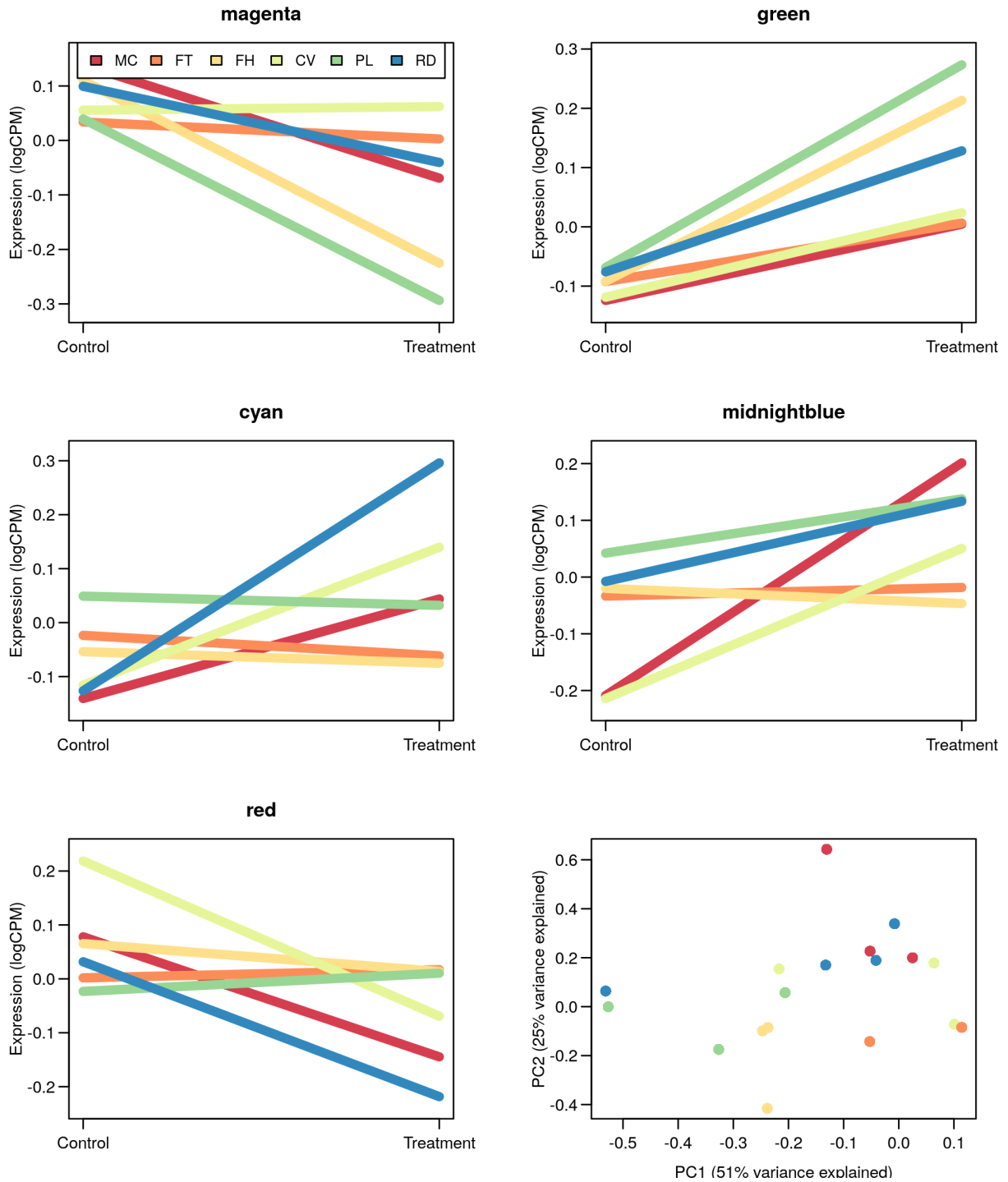
Figure 9. (A-E) Norm of reaction plots for module expression in each population and treatment for selected treatment-responsive modules with a strong G×E pattern and significantly enriched GO terms. Scale of y-axis varies. (F) PCA of individual module expression for all treatment-responsive modules, colored by home site.

Figure 10. Heatmap summarizing differential gene expression and module expression differences among sites. Populations are sorted by number of DE genes, and each cell for the gene module columns shows the average change in module expression between control and treated individuals for each site. Positive (red) values indicate upregulation under water stress treatment, negative (blue) values indicate downregulation.
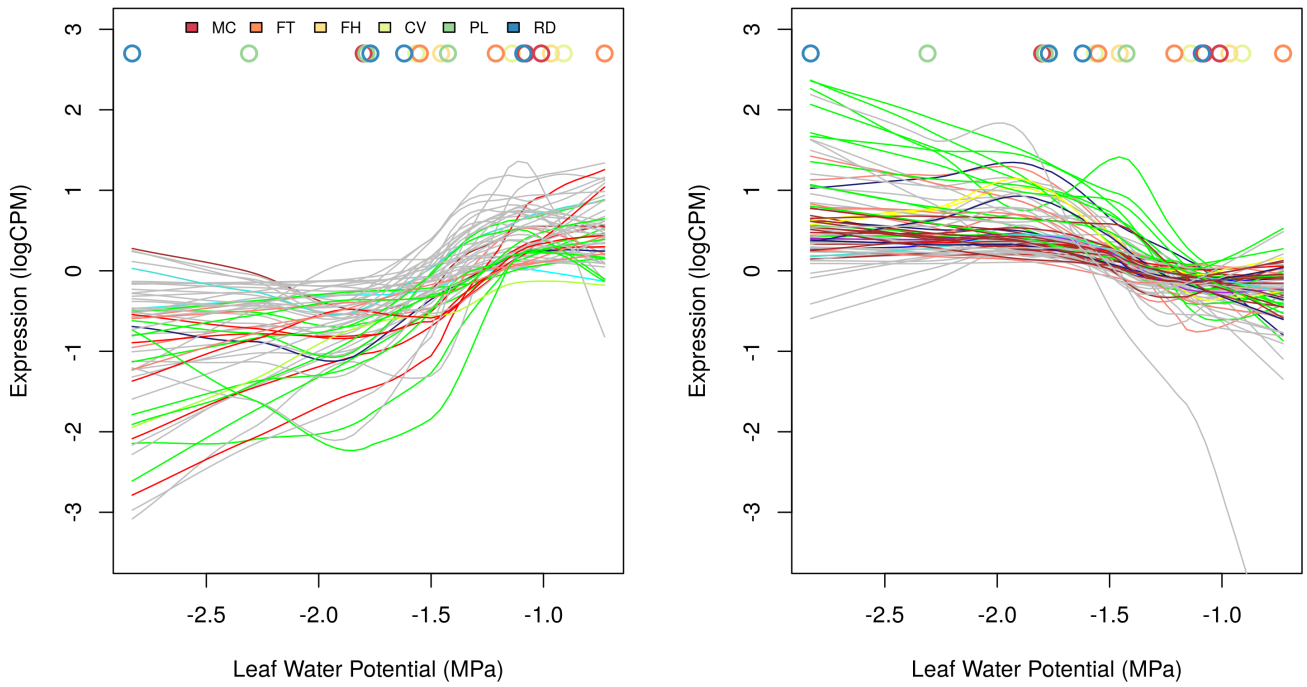
Figure 11. Smoothed lines showing the relationship of leaf water potential and gene expression for 19 seedlings in the water stress treatment. Genes shown had MIC p-values <0.001, and are sorted by positive (left, 62 genes) and negative (right, 77 genes) relationships. Line colors indicate the WGCNA module assigned to the gene (Table 6). Points at the top show the Ψ_{leaf} for each individual seedling, colored by home site.

Table 1. Summary of experimental design, with cells showing the number of seedlings per treatment per site. Both ecophysiology and gene expression data were collected for day 10, and only ecophysiology data were collected on day 20. Parenthesis indicate the number of seedlings which were sequenced when that number is different from the number measured for ecophysiological traits due to unsuccessful RNA extraction or sequencing.

Home Site	Day 10		Day 20	
	Control	Water Stress Treatment	Control	Water Stress Treatment
MC	3	4 (3)	4	4
FT	4 (3)	4 (3)	4	4
FH	4	4 (3)	4	3
CV	4	4	4	3
PL	4	4 (3)	4	4
RD	4	4	4	4

Table 2. Results of ANOVA comparing traits across treatments and home sites of seedlings for day 10 measurements. P-values were adjusted using Benjamini-Hochberg method; when p-values were significant before adjustment, both values are shown. Significant ($p<0.05$) results are in bold. Degrees of freedom (df) is different for some traits due to missing measurements for some individuals. 'Num.' = numerator, 'denom.' = denominator. NS = not significant.

Measurement	Site					Treatment					Site × Treatment				
	df (num.)	df (denom.)	F	p	p adj.	df (num.)	df (denom.)	F	p	p adj.	df (num.)	df (denom.)	F	p	p adj.
Height	5	35	3.688	**0.00873**	0.288	1	35	1.815	NS	NS	5	35	0.791	NS	NS
Largest Leaf Length	5	35	2.41	0.0559	NS	1	35	2.15	NS	NS	5	35	1.916	NS	NS
Largest Leaf Width	5	35	1.674	NS	NS	1	35	0.952	NS	NS	5	35	0.91	NS	NS
Largest Leaf Thickness	5	35	4.565	**0.00261**	0.0863	1	35	3.282	0.07865	NS	5	35	0.447	NS	NS
Average Leaf Thickness	5	35	2.852	**0.0291**	NS	1	35	2.256	NS	NS	5	35	0.577	NS	NS
Leaf Area	5	34	1.163	NS	NS	1	34	0.312	NS	NS	5	34	0.552	NS	NS
Leaf Dry Mass	5	35	1.101	NS	NS	1	35	1.516	NS	NS	5	35	0.473	NS	NS
Leaf Water Potential	5	35	0.433	NS	NS	1	35	3.115	0.0863	NS	5	35	1.081	NS	NS
Turgor Loss Point	5	35	2.479	0.0504	NS	1	35	21.551	**4.70E-05**	**0.00155**	5	35	3.117	**0.0198**	0.3261
Soil Mass	5	33	1.416	NS	NS	1	33	584.039	**<2e-16**	**<2e-16**	5	33	0.285	NS	NS
Soil Water Potential	5	34	0.99	NS	NS	1	34	7.724	**0.00881**	NS	5	34	0.705	NS	NS

10-day Measurements

Table 3. Results of ANOVA comparing traits across treatments and home sites of seedlings for day 20 measurements. P-values were adjusted using Benjamini-Hochberg method; when p-values were significant before adjustment, both values are shown. Significant (p<0.05) results are in bold. Degrees of freedom (df) is different for some traits due to missing measurements for some individuals. 'Num.' = numerator, 'denom.' = denominator. NS = not significant.

| | 20-day Measurements | | | | | | | | | | | | | | |
| Measurement | Site | | | | | Treatment | | | | | Site × Treatment | | | | |
	df (num.)	df (denom.)	F	p	p adj.	df (num.)	df (denom.)	F	p	p adj.	df (num.)	df (denom.)	F	p	p adj.
Height	5	34	13.086	**3.90E-07**	**1.29E-05**	1	34	2.121	NS	NS	5	34	1.725	NS	NS
Largest Leaf Length	5	34	2.298	0.0667	NS	1	34	0.032	NS	NS	5	34	2.005	NS	NS
Largest Leaf Width	5	34	1.004	NS	NS	1	34	0.389	NS	NS	5	34	2.092	0.0904	NS
Largest Leaf Thickness	5	34	3.013	**0.0234**	NS	1	34	4.303	**0.0457**	NS	5	34	1.027	NS	NS
Average Leaf Thickness	5	34	2.343	0.0624	NS	1	34	6.001	**0.0196**	NS	5	34	2.281	0.0684	NS
Leaf Area	5	34	0.522	NS	NS	1	34	0.411	NS	NS	5	34	0.751	NS	NS
Leaf Dry Mass	5	26	0.294	NS	NS	1	26	0.006	NS	NS	5	26	0.562	NS	NS
Leaf Water Potential	5	32	0.958	NS	NS	1	32	84.451	**1.72E-10**	**5.67E-09**	5	32	0.432	NS	NS
Turgor Loss Point	5	25	0.312	NS	NS	1	25	19.753	**0.000157**	**0.00519**	4	25	0.342	NS	NS
Soil Mass	5	34	0.434	NS	NS	1	34	103.25	**7.73E-12**	**2.55E-10**	5	34	0.203	NS	NS
Soil Water Potential	5	26	4.342	**0.00526**	0.0868	1	26	253.837	**6.23E-15**	**2.06E-13**	5	26	2.693	**0.0433**	NS

Table 4. Significantly enriched GO categories (p<0.05) from group of 1274 DE genes from the site + treatment model. P-values were adjusted for using the Benjamini-Hochberg method. Ontology refers to the three main groups for GO terms; molecular function (MF), biological process (BP), and cellular component (CC).

GO category	# genes in GO category for group	# genes in GO category total	Description	Ontology	P-value (adjusted)	Δexpression
GO:0009628	73	624	response to abiotic stimulus	BP	0.002	0.10
GO:0009408	16	62	response to heat	BP	0.004	0.23
GO:0080167	14	53	response to karrikin	BP	0.007	0.34
GO:0050896	157	1759	response to stimulus	BP	0.015	0.05
GO:0016853	23	137	isomerase activity	MF	0.015	0.03
GO:0006457	24	146	protein folding	BP	0.015	0.09
GO:0009266	28	183	response to temperature stimulus	BP	0.015	0.14
GO:0009687	5	7	abscisic acid metabolic process	BP	0.017	0.27
GO:0043288	5	7	apocarotenoid metabolic process	BP	0.017	0.27
GO:0042221	68	636	response to chemical	BP	0.022	0.08
GO:0010033	48	407	response to organic substance	BP	0.030	0.09
GO:0006714	5	8	sesquiterpenoid metabolic process	BP	0.032	0.27

Table 5. Significantly enriched GO categories (p<0.05) from group of DE genes between treatments for each site. Only MC and CV had significant DE genes that had significantly enriched GO terms. P-values were adjusted for each site using the Benjamini-Hochberg method. Ontology refers to the three main groups for GO terms; molecular function (MF), biological process (BP), and cellular component (CC). The average expression of all genes within a GO category was calculated for each sample, and these values for a given site/treatment combination were averaged, and the "Δexpression" is the treatment-control average (so positive numbers indicate higher expression on average in the water stress treatment).

Site	GO category	# genes in group	# genes total	Description	Ontology	p	Δexpression for site
MC	GO:0016614	13	146	oxidoreductase activity, acting on CH-OH group of donors	MF	0.009	0.46
	GO:0003824	128	4898	catalytic activity	MF	0.009	0.22
	GO:0034637	13	140	cellular carbohydrate biosynthetic process	BP	0.009	0.44
	GO:0005975	27	535	carbohydrate metabolic process	BP	0.009	0.29
	GO:0055114	36	941	oxidation-reduction process	BP	0.012	0.22
	GO:0016616	12	139	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	MF	0.012	0.45
	GO:0016491	33	858	oxidoreductase activity	MF	0.016	0.23
	GO:0019438	10	101	aromatic compound biosynthetic process	BP	0.016	0.60
	GO:0044262	15	214	cellular carbohydrate metabolic process	BP	0.016	0.36
	GO:0008652	11	128	cellular amino acid biosynthetic process	BP	0.016	0.32
	GO:0008152	145	5998	metabolic process	BP	0.016	0.20
	GO:0016229	7	51	steroid dehydrogenase activity	MF	0.016	0.55
	GO:0016053	15	230	organic acid biosynthetic process	BP	0.016	0.37
	GO:0046394	15	230	carboxylic acid biosynthetic process	BP	0.016	0.37
	GO:0005976	12	153	polysaccharide metabolic process	BP	0.021	0.43
	GO:0016051	13	177	carbohydrate biosynthetic process	BP	0.021	0.41
	GO:0003849	3	5	3-deoxy-7-phosphoheptulonate synthase activity	MF	0.021	2.04
	GO:0044283	16	275	small molecule biosynthetic process	BP	0.023	0.33
	GO:0048037	16	278	cofactor binding	MF	0.023	0.31
	GO:0033692	10	111	cellular polysaccharide biosynthetic process	BP	0.024	0.51
	GO:0016740	56	1744	transferase activity	MF	0.024	0.27
	GO:0000271	10	113	polysaccharide biosynthetic process	BP	0.025	0.51
	GO:0044264	11	136	cellular polysaccharide metabolic process	BP	0.025	0.46
	GO:0006725	11	146	cellular aromatic compound metabolic process	BP	0.025	0.47
	GO:0006520	15	263	cellular amino acid metabolic process	BP	0.032	0.21
	GO:0016020	67	2291	membrane	CC	0.038	0.27
	GO:0009698	6	45	phenylpropanoid metabolic process	BP	0.043	1.00
	GO:0006082	20	433	organic acid metabolic process	BP	0.043	0.27
	GO:0019752	20	433	carboxylic acid metabolic process	BP	0.043	0.27
	GO:0043436	20	433	oxoacid metabolic process	BP	0.043	0.27
	GO:0003854	6	49	3-beta-hydroxy-delta5-steroid dehydrogenase activity	MF	0.043	0.54
	GO:0033764	6	49	steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	MF	0.043	0.54

	GO ID						
	GO:0009073	5	30	aromatic amino acid family biosynthetic process	BP	0.046	0.57
CV	GO:0009536	40	1616	plastid	CC	0.001	0.2754191
	GO:0009507	39	1581	chloroplast	CC	0.001	0.2752552
	GO:0009532	15	293	plastid stroma	CC	0.002	0.480083
	GO:0009570	13	279	chloroplast stroma	CC	0.020	0.4805678
	GO:0044435	19	593	plastid part	CC	0.039	0.400711

Table 6. Number of genes in each WGCNA module (with percentage of total in parentheses). Modules for the significant (p<0.001) relationships with leaf water potential (Ψ_{leaf}) and turgor loss point (π_{TLP}) are also shown. Representative examples of significantly enriched GO terms for each module are also shown (NS = none significant). The "grey" genes are those that could not be assigned to a module.

Module	Number of genes	Genes related to Ψ_{leaf}	Genes related to π_{TLP}	Example enriched GO terms
black	375 (1.6%)	0 (0%)	0 (0%)	NS
blue	843 (3.6%)	0 (0%)	0 (0%)	Respiratory chain; mitochondrial membrane; photorespiration
brown	719 (3%)	16 (11.6%)	1 (4%)	Chloroplast; thylakoid; gene expression; oxidation-reduction process; photosynthesis
cyan	157 (0.7%)	1 (0.7%)	0 (0%)	Ribosome; translation; gene expression; protein folding
darkred	37 (0.2%)	0 (0%)	0 (0%)	NS
green	543 (2.3%)	22 (15.9%)	1 (4%)	Response to hormone; response to water deprivation; nucleic acid binding transcription factor activity
greenyellow	232 (1%)	1 (0.7%)	0 (0%)	Cell wall; biological regulation
grey	13466 (56.9%)	70 (50.7%)	23 (92%)	NS
grey60	120 (0.5%)	0 (0%)	0 (0%)	L-ascorbic acid metabolic process; hydrogen ion transmembrane transporter activity
lightcyan	138 (0.6%)	0 (0%)	0 (0%)	NS
lightgreen	86 (0.4%)	0 (0%)	0 (0%)	NS
lightyellow	75 (0.3%)	0 (0%)	0 (0%)	NS
magenta	303 (1.3%)	0 (0%)	0 (0%)	Photosynthesis; chloroplast thylakoid; response to abiotic stimulus; pigment biosynthetic process
midnightblue	152 (0.6%)	6 (4.3%)	0 (0%)	Aromatic compound biosynthetic process; response to UV; flavonoid metabolic process; oxidoreductase activity, acting on CH-OH group of donors
pink	364 (1.5%)	0 (0%)	0 (0%)	Chloroplast; thylakoid; photosynthesis; oxidation-reduction process; response to temperature stimulus
purple	232 (1%)	1 (0.7%)	0 (0%)	RNA-directed DNA polymerase activity; DNA replication
red	449 (1.9%)	7 (5.1%)	0 (0%)	Proteasome complex; protein catabolic process
royalblue	61 (0.3%)	0 (0%)	0 (0%)	NS
salmon	158 (0.7%)	10 (7.2%)	0 (0%)	Chloroplast; protein folding; seed development
tan	191 (0.8%)	0 (0%)	0 (0%)	NS
turquoise	4292 (18.1%)	2 (1.4%)	0 (0%)	Nucleic acid binding; protein transport; gene expression
yellow	690 (2.9%)	2 (1.4%)	0 (0%)	Plasma membrane; cell wall biogenesis; protein kinase activity
TOTAL	23683	138	25	

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