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Transcriptomics of Cotton Lines with Contrasting Drought Stress Response

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Abstract

Abiotic stresses affect cotton growth, yield, and development accounting for ~50% of yield reduction around the world. Drought is one of the most important abiotic stresses, however the molecular mechanism and the genes involved in drought tolerance or sensitivity have yet to be completely understood. Cotton is most widely used in the textile industry and is a source of edible oil. For commercial applications, high-yielding cotton that is tolerant to drought stress is highly valuable, and understanding the molecular mechanism would unlock the path to creating higher-yielding cotton that is stress tolerant. Breeding drought-tolerant varieties also leads to sustainable cotton production. In this research, genes that are differentially expressed were identified between two drought-tolerant and two drought-sensitive lines. These results provide a deeper understanding of the genes that play a crucial role in regulation, adaptation to drought stress, and potential targets for molecular breeding.

Keywords: Cotton, Transcriptomics, Drought, RNA sequencing

1. Introduction

Upland cotton (*Gossypium hirsutum*) is an economically important crop accounting for 90% of the overall cotton production (Yuan et al., 2018). It is an important source of fiber for the textile industry and cotton seeds serve as a source of edible oil with the by-products serving as cattle feed such as the meal and hulls. The United States produces about 35% of the cotton globally and is the largest exporter of cotton. Cotton is grown in diverse climatic conditions around the world and is subjected to frequent unfavorable abiotic stresses such as drought, salinity, and heat. According to reports, 57% of cotton worldwide is cultivated in water-deficit areas (World Resources Institute)

Drought is one of the major abiotic factors limiting agricultural production impacting the growth, yield, and fiber quality of cotton (Parida et al., 2007). Generally, drought stress severely restricts cotton growth and development, such as affecting plant height, leaf dry weight, stem dry weight, leaf area index, node number, fiber quality, canopy and root development (Loka et al., 2011). Drought occurs frequently during the boll-forming period in many cotton production regions, and cotton is most sensitive to water stress during this period (Loka and Oosterhuis, 2012). The frequency and intensity of drought will increase with the projected changes in global climate in the future (Giorgi and Lionello, 2008). In order to have sustainable cotton production, it is thus necessary to understand the impacts of drought on cotton and obtain varieties that are drought tolerant hence preserving or increasing yield. The ideal crop is one that requires less water but produces higher yields and better fiber quality. One way to do this is through breeding resistant varieties which is quite challenging due to the narrow genetic base of cultivated cotton species and sensitivity to various stresses. Another way is to understand the molecular mechanisms of drought's effect on cotton growth and modify the gene targets to get the desired phenotype.

Drought tolerance is a complex phenomenon because it is a multigenic system that is related to various morpho-physiological, biochemical, and molecular processes (Ullah et al., 2017). Drought induces plant responses including

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but not limited to altered gene expression patterns, accumulation of metabolites such as abscisic acid (ABA), and synthesis of specific proteins, namely hydrophilic proteins, reactive oxygen scavenging proteins, and chaperones (Ghodke et al., 2020). Drought stress causes stomata closure, which leads to decreased CO2 intake, affecting the rate of photosynthesis and consequently, reducing growth and yield (Chaves et al., 2003, 2009). Physiological impacts include an increase in photorespiration, oxidative damage to the chloroplast, and obstruction of adenosine triphosphate (ATP) synthesis that make the plant drought sensitive. Drought-tolerant plants typically increase the reactive oxygen scavenging species or accumulate sugars, amino acids, alkaloids, polyols, and inorganic ions for osmotic adjustment and in turn maintain photosynthesis and other drought-related processes leading to maintaining and improving yield.

Plants including cotton respond or adapt to various stresses, and transcriptional modulation is one of the most important ways that induce or repress gene expression in plants under biotic and abiotic stresses. Transcriptomics is the genome-wide identification of gene expression and RNA sequencing enables the identification of transcripts for the expression of all genes in a single organism under precise physiological conditions or treatments, facilitating global analysis of gene functions and structures. There have been studies published on molecular mechanisms of stress response in diverse cotton species primarily using transcriptomics which facilitated the identification of key genes to be differentially expressed under stress. In *Gossypium hirsutum*, root transcriptomics indicated 1530 transcripts to be co-expressed in natural rain-fed and well-watered (WW) cotton in the field. In wild cotton *Gossypium darwanii*, RNA sequencing of leaf tissue identified 58,961 genes differentially expressed between seedlings with and without drought stress (Xu et al., 2022). RNA sequencing enabled the identification of 6968 transcripts that were differentially expressed with statistical significance in three cotton species *Gossypium hirsutum*, *Gossypium arboreum*, and *Gossypium barbadense* (Hasan et al., 2019). Upland cotton was studied in a hydroponic system with polyethylene glycol (PEG) to simulate drought stress to conduct network analysis and identify key pathways in stress adaptation (Zheng et al., 2022).

In this study, two drought-tolerant (DT) and two drought-sensitive (DS) lines from *Gossypium hirsutum* were selected to be analyzed by RNA sequencing. The lines were selected based on preliminary research indicating differences in yield measurements under water-limiting and well-watered conditions. The plants were subjected to two levels of stress: moderate drought (MD) and severe drought (SD) involving different levels of water limitation. The study also included a recovery period to assess how the plants adapt to water availability after the deficit period. This study aided in the identification of transcripts and shed light on the molecular processes likely involved in drought tolerance of cotton and contributing to yield.

2. Material and Methods

2.1 Genotypes

Four genotypes were chosen based on preliminary research done internal to the lab in water-limited and full irrigation conditions (unpublished data). Performance of the lines based on percent yield estimate difference in water-limited compared to well-watered conditions were considered for line selection. Lines 1179 and 1896 showed a mean of 10% increase yield estimate in water-limited conditions and lines 1189 and 3031 showed a mean of 40% reduction in yield in water-limited conditions. 1179 and 1896 were then chosen as the drought tolerant and 1189 and 3031 as the drought susceptible genotypes for this study.

2.2 Plant growth

Cotton seeds were potted in 4.5-inch pots with soil consisting of peat moss and vermiculite mixture, grown at 24°C and 45% relative humidity, and watered twice a week (Figure 1). Medium-sized pots were selected to ensure that the water limiting conditions were uniform throughout the pot, which would not be possible in smaller pots. The plants were arranged in a randomized design with four replications. When the plants had 5-6 leaves, drought treatment was initiated. The moisture content was measured with a Field Scout Soil Moisture meter. Prior to establishing water limited conditions, the soil had 35-40% Volumetric Water Content (VWC) which marked our well-watered control.

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When the moisture reached 11-12% VWC, this constituted the moderate drought (MD) group. The young fully expanded leaves were sampled by flash freezing in liquid nitrogen, placed in dry ice, and kept frozen at -80℃ till RNA extraction. The pots were not watered for another 24 hours when the soil moisture content reduced to 7-9% VWC and the soil was visibly dry. Young leaves sampled at this stage belong to the severe drought (SD) group. The plants were then irrigated fully, and leaves were sampled again the next day when the moisture content was 35-40% VWC to constitute the recovery group. Sampling was done in triplicates to account for biological replicates in this experiment. Frozen leaves were ground and stored at -80℃.

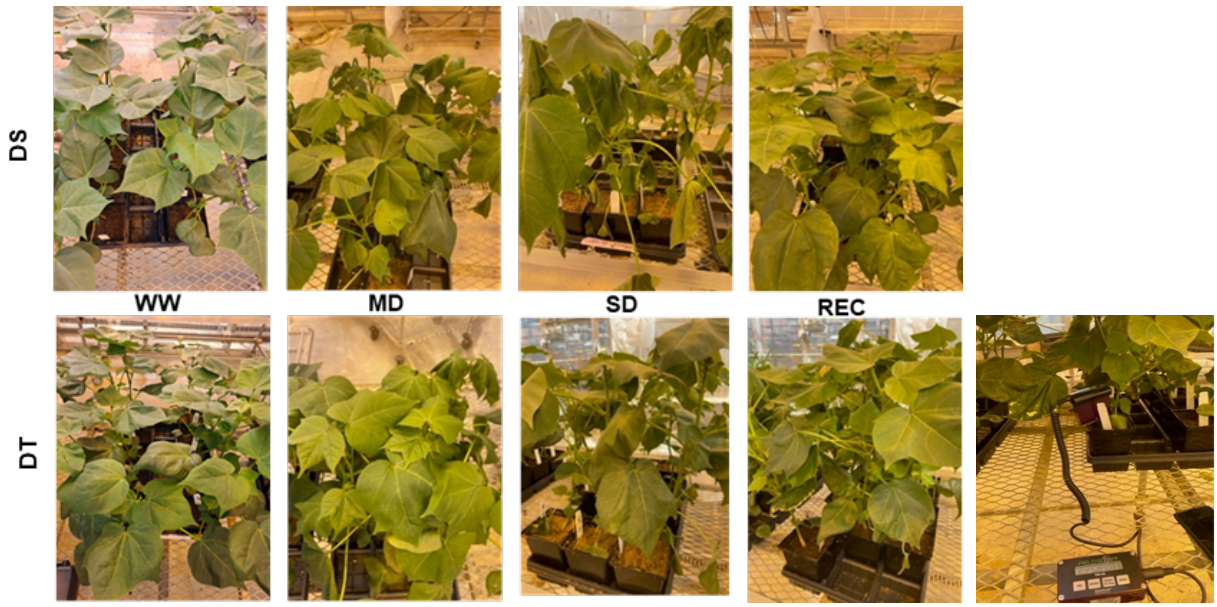

Figure 1. Cotton lines in the well-watered (WW), moderate drought (MD), severe drought (SD), and Recovery (Rec) period. The panel on the right shows the FieldScout Soil Moisture meter.

2.3 RNA Extraction

100 mg of tissue was used for RNA extraction using the Spectrum Plant Total RNA kit as per the manufacturer's instructions. The tissue was first lysed, DNAse treated, washed and RNA was eluted in 50ul of elution buffer. RNA quantity and quality were checked on nanodrop for A260/280 and A260/230 values and on an Agilent tapestation. The ratio of absorbance at 260nm and 280nm indicates the purity of RNA. A ratio close to 2 indicates that the RNA is pure. If the ratios are lower, it indicates contamination with proteins or remnant reagents from the extraction that absorb at 280nm. The ratio of absorbance at 260 nm and 230 nm is a secondary measure of RNA quality and a range of 2.1-2.2 indicates pure RNA and lower ratios indicate contaminants that absorb at 230nm.

2.4 Library Preparation and Sequencing

mRNA was enriched from total RNA using oligo dT beads that attach to the polyA tails of total RNA to select for mRNA. mRNA was reverse transcribed to cDNA using Reverse Transcriptase enzyme. cDNA was end prepped for adaptor ligation and indexing with barcodes followed by polymerase chain reaction (PCR) amplification for 12 cycles. The libraries were purified and then quantified using Qubit and normalized to 2nM and sequenced on a Nextseq 2000 sequencer with 2% PhiX for 300 cycles.

2.5 Data Analysis

Demultiplexed data was checked for quality using FastQC which is a quality control application for sequencing data (Andrews S, 2010). Four main parameters were used as indicators of quality. Per base sequence quality indicates

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the quality value across all bases at each position. Per sequence quality score indicates if subset of sequences will have low quality values. Per tile sequence quality indicates quality in each tile across all the bases to detect loss of quality in any part of the flowcell used for sequencing. Per base N content indicates the number of bases called as N if the sequencer is unable to make an accurate call for each base as A,T,G or C. The data was then mapped to the cotton genome (*Gossypium hirsutum* v1.1) using the mapper STAR (Spliced Transcript Alignment to a Reference) that identifies the location of each read in the sequencing data on the reference genome (Dobin et al, 2013). The BAM (binary alignment map) file from mapping and a GFF (general feature format) file with gene models were inputted to htseq_count which counts the number of aligned reads overlapping its exons for each gene. R was used to plot gene names and plot data, and DESeq was used for differential expression analysis. Pairwise comparison was done using the read counts from each of the treatment groups within each line. The output generated gene lists with p-value and fold change for each gene. DESeq uses the Wald test to calculate the p-value and the null hypothesis is that there is no differential expression between the sample groups. If the p-value is very small ($p<0.05$) then the null hypothesis is rejected indicating that the change in expression observed in the sample groups is significant (Anders S, Huber W, 2010). Fold change indicates if a gene is upregulated or downregulated. It is calculated as the ratio of the difference in read counts between the treatment and the control groups over the control and reported in the logarithmic scale to the base 2. A log2 fold change (FC) of greater than 1.5 is typically considered significant. A fold change value of 2 was used here for increased stringency and values greater than 2 indicate upregulation in expression and less than -2 indicate downregulation.

3. Results

3.1 Sequencing Statistics and quality check results

RNA measured on nanodrop resulted in an A260/230 value of 2.0 and an A260/280 value of 2.1 indicating that RNA was very pure. Library distribution was between 300-700 bp (base pair) and 10 million reads per library was generated across a total of 48 samples, of which an average of 9.3 million reads mapped uniquely to the genome indicating a 90% mapping rate. FastQC indicated high quality of the raw data: per base sequence quality >30; per tile sequence quality 0.3-0.4, per sequence quality score >30 and per base N content is <1%. If the per base sequence quality is >28, per tile sequence quality > 0.1, per sequence quality score of >27 and per base N content is less than 5%, then the quality is regarded as high based on the threshold. 88-92% of the reads are uniquely mapped to the genome. 10% of the reads were multi-mapped and 0.2% did not map to the genome indicating good quality sequencing data (Figure 2).

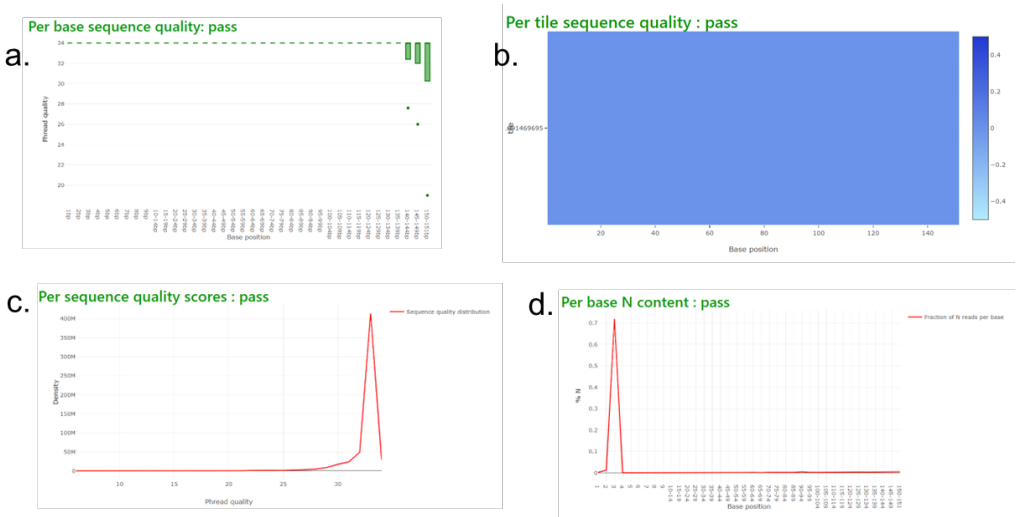

Figure 2. Quality check of the sequencing data. a) Per base sequence quality b) Per tile sequence quality c) Per sequence quality scores d) Per base N content

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Principal Component Analysis indicated that the samples clustered by treatment indicate that the treatment effect was stronger than the genotype effect (Figure 3). Well-watered and recovery groups clustered together indicating that the plants reverted to their well-watered state in the recovery period. The leaves after the recovery phase also appeared visually healthy like that of the initial well-watered state. Moderate drought and severe drought samples clustered distinctly from each other and from the control group also validating the correctness of the water-limiting conditions established in the glasshouse.

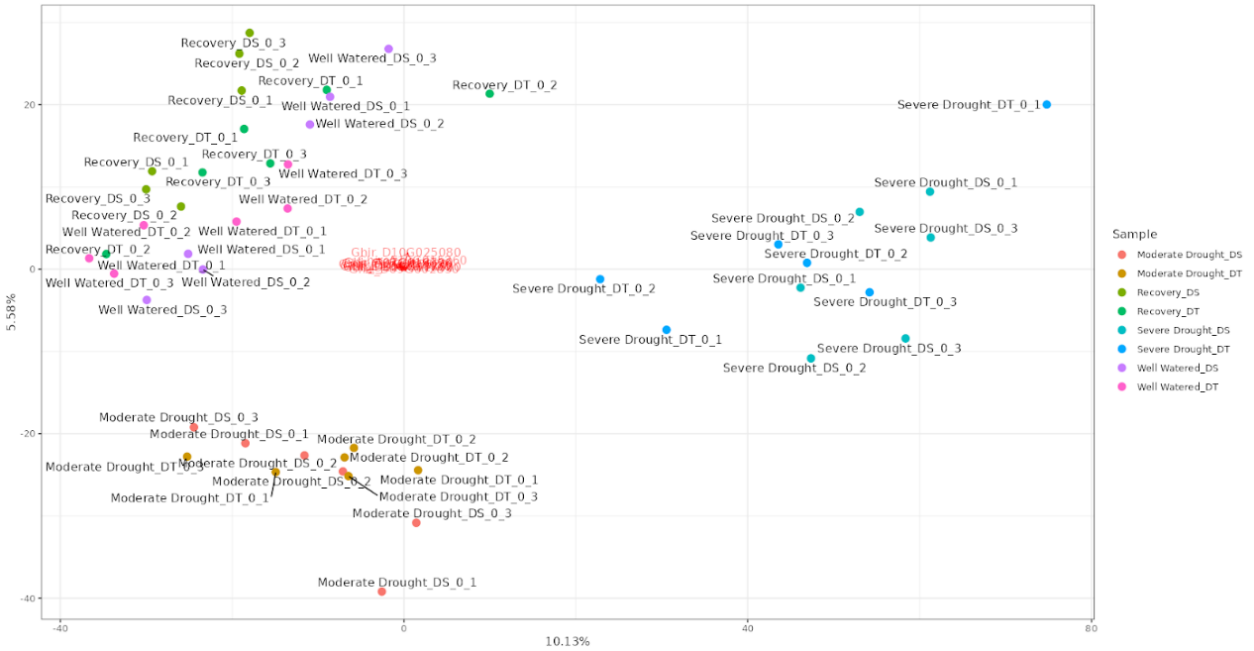

Figure 3. Principal Component Analysis (PCA) plot

3.2 Differential Gene Expression

Differential expression was performed between each drought treatment compared to well-watered control in each line (Table 1). Total number of genes that were differentially expressed included thousands of genes at $p<0.05$. The genes were further filtered to select for those that are changing more than 2-fold indicating hundreds of genes in each line with severe drought having more differentially expressed genes than moderate drought for each line.

Differential Gene expression was performed between the drought-sensitive (DS) and the drought-tolerant (DT) lines and between the drought severity treatments (moderate vs severe) within each group (Figure 4). 466 genes commonly changed in expression between MD and SD in lines 1189 and 197 genes in 3031 (DS lines). 256 genes were commonly changed in expression between MD and SD in lines 1179 and 293 genes in 1896 (DT lines). 1725 genes were commonly changed in expression among the severe drought and 267 in MD (DS lines). 870 genes were differentially expressed in the SD group and 314 genes in the MD group (DT lines). This indicates a strong drought response in all genotypes and the number of differentially expressed genes increased with the severity of the drought.

Table 1. Total number of differentially expressed genes that are statistically significant for each line and treatment type.

Line_treatment	# genes ($p<0.05$)	# genes ($p<0.05$;FC>2)	Line_type
1189_MD	6415	799	DS
3031_MD	8377	1088	DS
1179_MD	5421	759	DT
1896_MD	7996	535	DT
1189_SD	16101	3015	DS
3031_SD	14542	2362	DS
1179_SD	6258	1037	DT
1896_SD	19360	4799	DT

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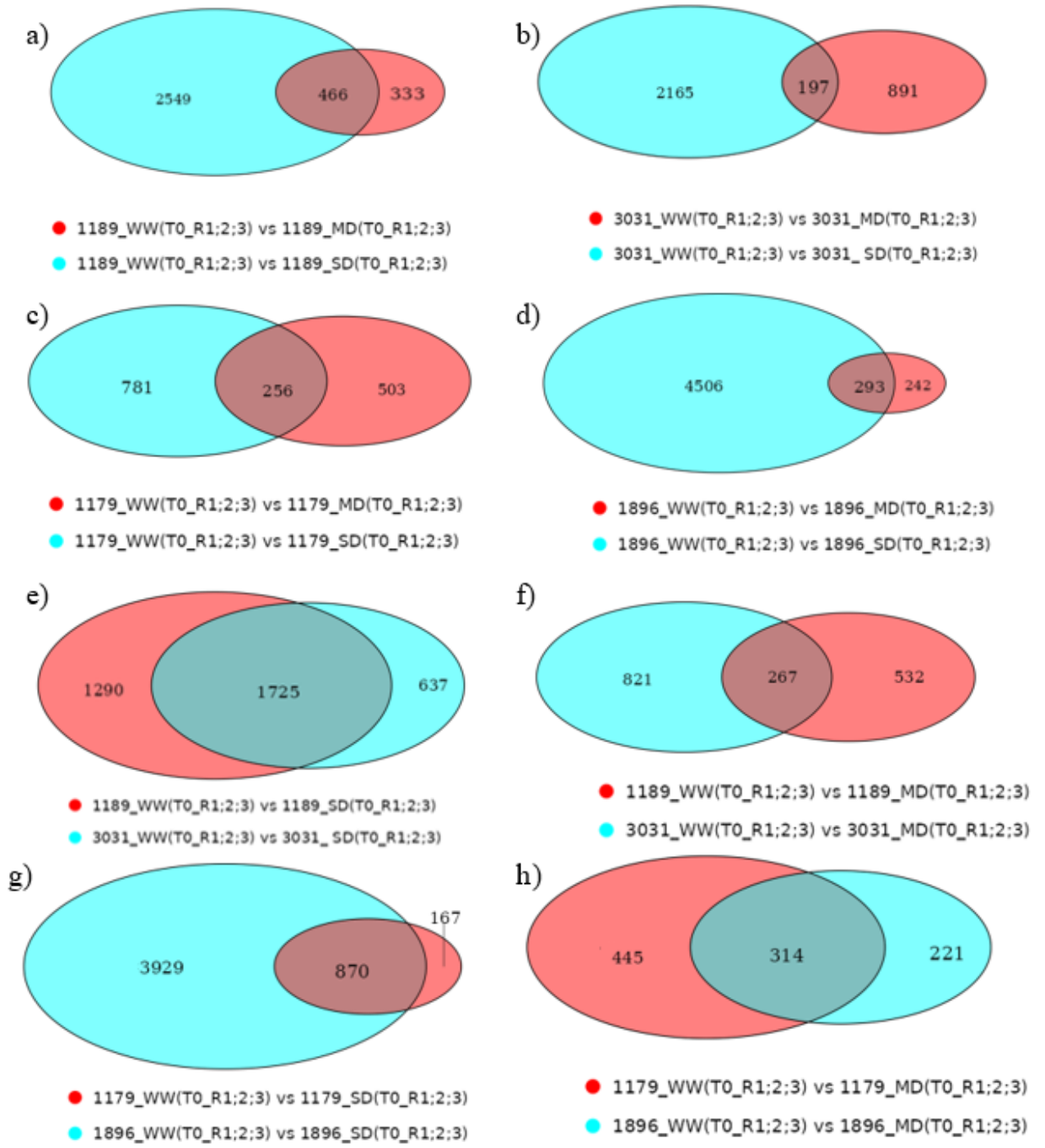

Figure 4. a) DS line 1189 MD vs SD b) DS line 3031 MD vs SD c) DT line 1179 MD vs SD d) DT line 1896 MD vs SD e) DS lines 1189 vs 3031 SD f) DS lines 1189 vs 3031 MD g) DT lines 1179 vs 1896 SD h) DT lines 1179 vs 1896 MD

The genes that were upregulated in SD indicate genes with a potential role in drought tolerance and regulation of stress response in the tolerant plants. NAC domain protein is a classic transcription factor involved in stress response and was upregulated 6-fold in SD. NAC transcription factors are relevant in abscisic acid-dependent and independent pathways in drought stress signaling. Overexpression of NAC genes in rice conferred enhanced drought and salt tolerance (Zheng et al., 2009). OsNAC10 increased tolerance to drought and provided increased grain production in rice (Jeong et al., 2010). Drought-induced unknown protein 1 (DIUP1) was upregulated 10-fold in this study.

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Overexpression of DIUP1 from alfalfa in Arabidopsis resulted in increased tolerance to drought, with higher seed germination, root length, fresh weight, and survival rate than in wild-type plants (Luo et al, 2023). Sugar transporter protein was upregulated 12-fold in severe drought conditions. Sugar transporter proteins are key determinants of the influx/efflux of various sugars and their metabolite intermediates that support the plant growth and developmental process. Abiotic stress, especially drought stress-mediated-injury is known to result in reprogramming of sugar distribution across the cellular and subcellular compartments (Kaur et al., 2021). Cytochrome p450 was upregulated 6-fold and it belongs to the family of oxidoreductase enzymes that are known to play a role in plant stress response. Galactinol and raffinose synthase were upregulated 10-fold in SD conditions. Raffinose and its precursor galactinol accumulate in plant leaves during abiotic stress-knockout in maize has been shown to modulate drought sensitivity (Li et al, 2020).

A lot of the drought-responsive genes that are significantly differentially expressed were common in both DT lines and DS lines. The difference was in the level of their upregulation in DT vs DS lines. Genes that were upregulated to a higher extent in DT lines and greater than 4-fold were investigated further (Figure 5).

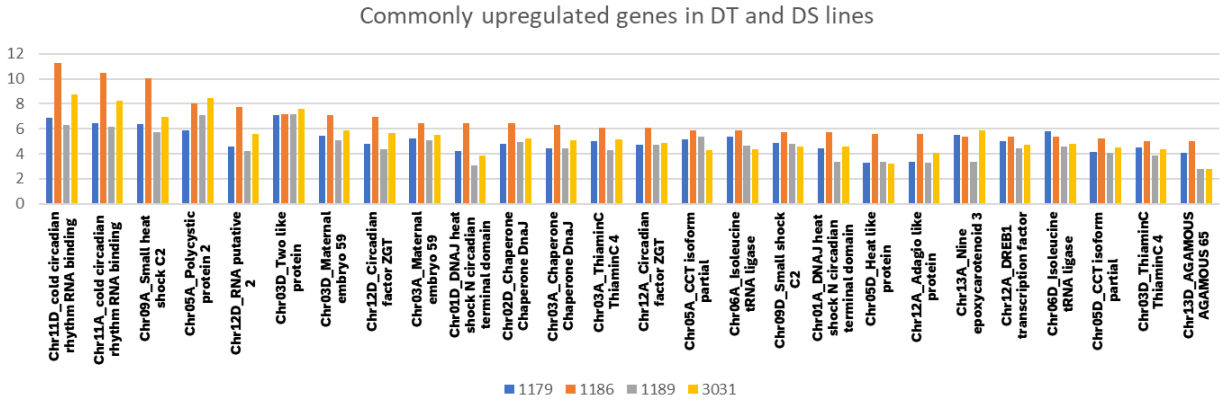

Figure 5. Commonly upregulated genes in DT (1179;1896) and DS (1189;3031) lines

These included cold circadian rhythms RNA binding-like (CCR-like) protein, heat shock protein, dehydration responsive element binding (DREB1) transcription factor, and agamous 65. These genes have supporting evidence in literature for their likely role in abiotic stress response regulation in other plant species. Cold-induced RNA-binding proteins regulate circadian gene expression by controlling alternative polyadenylation. CCR-like proteins from cotton when overexpressed in tobacco were shown to confer tolerance to abiotic stress (Gurusamy et al., 2015). Heat shock proteins are classic examples of genes that are upregulated in plant stress response. Overexpression of soybean DREB1 has been shown to confer tolerance to water stress in field wheat (Zhou et al., 2020). Agamous-like proteins such as AGL22 have been shown to regulate a transcriptional network during drought stress, linking changes in primary metabolism and the initiation of stress responses in Arabidopsis (Wang et al., 2018). The genes that were commonly downregulated include LHY-like proteins, aquaporins, glutathione transferase, and inositol 3 phosphate synthases. LHY proteins are known to negatively control drought tolerance in soybean and aquaporins regulate water homeostasis in plants. There were no uniquely expressed genes identified in either group that were above the 2-fold threshold indicating that it is the regulation of expression of multiple genes and or pathways that are conferring adaptation of the plant to stress.

3.3 Gene Ontology (GO) Enrichment Analysis

Enrichment analysis was conducted to identify the functional and biological significance of the upregulated genes. The transcripts were grouped into three categories based on the GO annotation: Cellular Component, Biological process, and Molecular Function ($p<0.05$).

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In DT lines, the Biological Process category involved enrichment in positive regulation of circadian rhythm (GO:0042753), protein dephosphorylation (GO:0006470), biosynthetic process for thiamine, (GO:0009228), proline (GO:0006562) and galactose metabolic process (GO:0006012). The Cellular Component involved enrichment in the cell wall (GO:0005618), nucleosome (GO:0000786), extracellular region (GO:0005576), and CCAAT-binding factor complex (GO:0016602) were enriched in DT lines. Molecular Function involved enrichment in cation binding (GO:0043169), DNA binding transcription factor (GO:0003700), protein serine/threonine phosphatase activity (GO:0004722), inositol 3 phosphate synthase activity (GO:0004512) were enriched in DT lines.

The DS lines showed similar enrichment in the Chemical Component and Molecular Function categories when compared to the DT lines. The Biological Process includes regulation of DNA replication (GO:0006260), negative regulation of DNA helicase activity (GO:1905775), systemic acquired resistance (GO:0009627), initiation of DNA replication (GO:0006270) that were highly enriched in DS lines. DS lines showed a pronounced enrichment of DNA replication and enzymes involved in the process regulating stress response compared to DT.

4. Discussion

Two contrasting drought-responsive genotypes were analyzed by transcriptome sequencing under 2 drought conditions (moderate and severe drought). More than 3000 genes were differentially expressed in the DT and DS genotypes. Recovery treatment enabled regaining of the leaf health and the gene expression levels reverted to the WT state. Moderate drought is more reflective of a field drought condition as severe drought is an extreme effect not regularly happening in the field. The severe drought treated plants looked visually stressed as well. Key genes were identified that are likely contributors to conferring drought tolerance in cotton. Quantitative Reverse Transcription Polymerase Chain Reaction (qRTPCR) can be used to confirm the identified gene targets in the lab. This would involve the RNA used for sequencing to be first reverse transcribed to cDNA and primers specific to the genes used for PCR. Because the primers and probes are fluorescently labeled, the amount of amplified product would be measured using fluorescence in each PCR cycle. Validation of the genes is not essential by additional wet lab techniques for all targets identified by RNA sequencing, especially with the progress of the sequencing technology and inclusion of biological replicates. However, such validation experiments could benefit the genes with low expression differences such as those with less than 2-fold (Coenye 2021).

The genes identified here provide important targets for molecular breeding and potential use for increasing yield potential. Gene overexpression in cotton for functional analysis of key genes would unravel their role in stress response and contribution to stress tolerance in certain genotypes. This can be achieved by transgenic technologies that include amplification of the gene sequence, cloning into a plasmid vector, and transformation of cotton plant with the Agrobacterium to integrate the target DNA into desired genotypes. These plants can then be tested in well-watered and water limiting conditions to check for yield performance of these transgenic lines. The genes can be introduced in this way to any genotype to improve drought tolerance or combine with other desirable phenotypes existing in the recipient line used to create transgenics.

One of the limitations in this study is that the experiments were conducted in a controlled environment in pots instead of the field. The variability of the field environment cannot be exactly simulated in the controlled environment. In the field, the drought effect is typically in the moderate drought range and seasonal variability cannot be excluded. In the current set up for the study, the primary factor that is changing is the water levels. One way any additional variability was by having 3 biological replicates for each condition and ensuring the selection of statistically significant transcripts. Follow-up studies of these lines in the field would be useful for the validation and refinement of identified targets. In this study, leaf tissue was tested and expanding the tissue sources to include the roots which are responsible for water uptake will help gain a more holistic understanding of the molecular mechanism of drought response and identify potentially more targets. One developmental stage was picked which is in the transition of the vegetative and the reproductive stage as that mimics the timing of maximum stress in nature for cotton plants and this stage is known to be physiologically important for stress adaptation. More vegetative and reproductive stages of tissue sampling could be added for a broader understanding.

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The category of Biological Process from Gene Ontology Enrichment Analysis is a strong lead from this study due to the differences between the DT and DS lines. The positive regulation of circadian rhythm is enriched in DT lines, and a cold circadian rhythm RNA binding like (CCR-like) Gm protein was identified as highly differentially expressed in the DT lines compared to DS lines. The ThiaminC gene upregulated more than 4-fold belonging to the biosynthetic process for thiamine is an interesting lead from this study. In a recent paper, foliar application of thiamine on pea plants has enabled the plants to withstand drought stress (Kausar et al., 2023). Recently, ThiaminC was also identified to be induced under stress along with thiamine deficiency in cotton roots and exogenous application of thiamine restored the deficiency induced during stress (Li et al., 2022). The third category which is interesting to follow up is the galactose metabolic processes; galactinol and raffinose synthase was identified as the genes upregulated in drought stress. Raffinose and its precursor galactinol have been identified to accumulate in the leaves of plants during stress. Raffinose synthase transfers the galactosyl group from galactinol to sucrose to catalyze the formation of raffinose. Mutant maize plants lacking raffinose synthase completely lacked raffinose and hyper-accumulated galactinol making them more sensitive to drought stress. Maize raffinose synthase when overexpressed in Arabidopsis plants enhanced drought stress tolerance through either raffinose synthesis or galactinol hydrolysis, depending on sucrose availability in plant cells (Li et al., 2020).

In conclusion, genes that are differentially expressed in contrasting cotton genotypes with different stress tolerance have been identified in this research study. The genes involved in thiamine metabolism have also been reported to be enriched in a drought resistant line of Upland Cotton (Zheng et al., 2022). This indicates both the importance of the thiamine metabolism pathways in drought tolerance of *Gossypium hirsutum* and the probability of genotype independence for drought tolerant varieties as it is enriched in the 2 drought tolerant lines used for this research study as well. While few common gene functions have been identified based on previous research in the cotton field such as thiamine metabolism and heat shock proteins, some new genes and pathways have been identified here such as cold circadian rhythm, galactose metabolism, and agamous-like proteins. These genes and the processes they are involved in cotton demonstrate strong targets for molecular breeding and this study has enhanced our understanding of the molecular basis of cotton drought stress response.

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