Comparative Analysis of Alfalfa (Medicago Sativa L.) Seedling Transcriptomes Reveals Genotype-specific Drought Tolerance Mechanisms

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Research article

Keywords: Alfalfa, Genotype, Drought stress, Transcriptome, Leaves, Roots

Posted Date: October 6th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-75482/v1

Abstract

Background

Drought is one of the main abiotic factors that affect alfalfa yield. The identification of genes that control this complex trait can provide important insights for alfalfa breeding. However, little is known about how alfalfa responds and adapts to drought stress, particularly in cultivars of differing drought tolerance.

Results

In this study, the drought-tolerant cultivar Dryland ‘DT’ and the drought-sensitive cultivar WL343HQ ‘DS’ were used to characterize leaf and root physiological responses and transcriptional changes in response to water deficit. Under drought stress, Dryland roots (DTR) showed more differentially expressed genes than WL343HQ roots (DSR), whereas WL343HQ leaves (DSL) showed more differentially expressed genes than Dryland leaves (DTL). Many of these genes were involved in stress-related pathways, carbohydrate metabolism, and lignin and wax biosynthesis, which may have improved the drought tolerance of alfalfa. We also observed that several genes related to ABA metabolism, root elongation, peroxidase activity, cell membrane stability, ubiquitination, and genetic processing responded to drought stress in alfalfa. We highlighted several candidate genes, including sucrose synthase, xylan 1,4-beta-xylosidase, primary-amine oxidase, and alcohol-forming fatty acyl-CoA reductase, for future studies on drought stress resistance in alfalfa and other plant species.

Conclusions

In summary, our results reveal the unique drought adaptation and resistance characteristics of two alfalfa genotypes. These findings, which may be valuable for drought resistance breeding, warrant further gene functional analysis to augment currently available information and to clarify the drought stress regulatory mechanisms of alfalfa and other plants.

Highlights

- We assembled leaf and root transcriptomes of drought-tolerant and drought-sensitive alfalfa cultivars under control and drought conditions.
- Under drought, the drought-tolerant cultivar exhibited more differentially expressed metabolites than the drought-sensitive cultivar.
- Genes associated with carbon metabolism, amino acid metabolism, hormones, and secondary metabolites were differentially expressed under drought stress.
- The drought-tolerant cultivar exhibited higher expression of genes encoding sucrose synthase, xylan 1,4-beta-xylosidase, primary-amine oxidase, and alcohol-forming fatty acyl-CoA.

Background

Drought is one of the most important environmental stresses in today's agricultural landscape, and it exacerbates the damage caused by other abiotic stresses such as heat, salt, and cold[1]. Widespread water pollution and global climate change have increased the frequency of water deficits[2]. Drought stress alters various plant biochemical and physiological processes, including those related to photosynthesis[3], glucose metabolism[4], and secondary metabolism [5], thereby inhibiting plant growth and yield. Plants use multiple mechanisms to respond to drought stress, and there are therefore many candidate pathways through which stress tolerance can be engineered and enhanced. For example, early flowering[6] and changes in the root-to-shoot dry mass ratio reduced root and shoot growth[7] and decreased the leaf expansion rate[8]. Adding to the complexity, crops respond quite differently to drought stress depending on their developmental stage, and drought can occur at different times during the growing season.

Alfalfa (*Medicago sativa* L.) is an important leguminous forage crop grown worldwide. Its deep root system not only prevents soil erosion but also improves soil fertility [9]. Alfalfa is also a promising crop for use as a bioenergy feedstock[10]. Compared with other crops, alfalfa is relatively tolerant to drought[11]. Although alfalfa varieties can adapt to water-limited regions, water deficits still have a negative impact on productivity[12]. Therefore, improving the drought resistance of alfalfa and increasing its potential as a forage remain the focus of many research groups[13, 14]. The benefits of biotechnology in improving crop yields in stressful environments have recently become evident with the first report of improved drought tolerance in field crops[15]. Recently, many transgenic alfalfa lines with enhanced tolerance to drought stress have been produced[16-18] through the manipulation of transcription factors (TF, Alfin1, GsWRKY20, MsWRKY33, WXP1), oxidases (CodA, MnSOD, MsALR, AtNDPK2, AVP1, EsMcsu1MsZEP), and other genes (MicroRNA156, SPL6, SPL12 and SPL13, IbOr GsZFP1, ZxNHX/ZxVP1-1). Transcriptional profiling in plants with drought-tolerant and drought-susceptible genotypes provides greater insights into the complexity of plant responses to drought stress at the molecular level[19-21]. Transcriptome profiling data from alfalfa have been reported in several microarray studies and, more recently, RNA-seq studies[22-25]. However, alfalfa is a heterogeneous synthetic population[26]. This trait makes it a genetically complex species, recalcitrant to direct genomics studies. Although some specific genes have been identified, the heterogeneous genetic backgrounds of tolerant and susceptible germplasms often obscure the relationship between genetic variation and drought tolerant phenotypes; thus, using the same alfalfa genetic background is very important in the identification of genes involved in drought stress. Furthermore, the transcriptional regulatory network of the water stress response in alfalfa remains unstudied.

Here, we used cloned populations with the same genetic background to screen out drought-tolerant and drought-susceptible genotypes through many long-term indoor and outdoor experiments. Our objectives were to identify drought-responsive differentially expressed genes and to investigate the molecular and physiological mechanisms of adaptation to drought stress in alfalfa using physiological experiments and RNA-seq data. The resulting drought tolerance candidate genes can be subjected to further functional characterization and used for genetic engineering or breeding new drought-tolerant cultivars for use in arid environments.

Results

Preliminary drought stress experiment

To investigate the transcriptional response of alfalfa to drought stress, we chose two varieties with different drought tolerances and performed RNA-seq transcriptome profiling. When alfalfa seedlings were grown with restricted water availability, the two varieties showed different phenotypes. The drought-tolerant variety DT showed relatively normal growth, whereas the drought-sensitive variety DS appeared to wilt (Fig. 1A). To choose a suitable sample collection time, we first selected six samples for use in a preliminary 6-d drought experiment. We observed that stem growth rate decreased significantly under drought stress; it declined more in DS than in DT, even shrinking on the fourth day (Fig. 1C). We therefore chose 4 d as the stress duration for the full experiment. DT had a greater root length than DS (Fig. 1B), and there were no significant differences between the varieties after drought stress. We also monitored ion changes under drought conditions. Levels of Ca^{2+} increased in roots but decreased in leaves after drought stress, and there was greater Ca^{2+} accumulation in DSR. DT had higher K^{+} content than DS, and K^{+} content decreased in DTR and DTL under drought stress. In DS, drought caused no significant changes in roots but a small increase in leaves. (Fig.1D, E).

Overview of transcriptome changes in two alfalfa genotypes under drought stress

To investigate the molecular responses of alfalfa to drought stress, we compared gene transcription of seedlings under drought and control conditions. A total of 73,059 unique genes were identified and quantified by RNA-seq. We used thresholds of $P < 0.05$ and ≥2-fold expression difference to identify genes whose expression differed significantly between control and drought treatments. Drought stress resulted in more transcriptional changes in DS than DT in comparison to normal growth conditions. Interestingly, there were more DEGs in DT roots than DS roots but more DEGs in DS leaves than DT leaves (Fig. 2A).

The differing transcriptome profiles of DT and DS under drought stress suggested that DEGs between the two cultivars were also associated with plant characteristics other than drought stress responses. Therefore, we first compared the DEGs across genotypes and organs and found that 395 DEGs were genotype- and organ-independent (Fig. 2B). Based on KEGG analysis, these DEGs were enriched in members of the carbohydrate metabolism, amino acid metabolism and signal transduction pathways (Supplementary Data Fig 1). In addition, most genes that were differentially expressed in both organs belonged to the category of plant hormone signal transduction (Supplementary Table 2). Another highly represented process was biosynthesis of amino acids. Within this pathway, Arabidopsis histidine kinase 2/3/4 (a cytokinin receptor) was upregulated only in DTR. GO term analysis indicated that two terms were significantly enriched: ABA-activated signaling pathway and response to water deprivation (Supplementary Data Fig. 2). Expression trends were similar for most of those genes, and all of them belonged to the biological process GO category. By contrast, a gene encoding the transcription factor KUA1 OS, which participates in plant hormone signal transduction, was upregulated only in DTR (Fig. 4).

Second, we compared organ-specific DEGs. In roots of both genotypes, the most significant difference in gene enrichment was in plant hormone signal transduction (Supplementary Data Table 3). Among these genes, 5'-AMP-activated protein kinase (AMPK) was slightly upregulated in DTR but significantly downregulated in DSR, and the expression of genes associated with histidine and ethylene increased more in DTR than in DSR. Expression of genes encoding SAUR family protein, mitotic-specific cyclin-B1, and NADPH decreased more in DTR than in DSR. Among lipid metabolism genes, those encoding 3-ketoacyl-CoA synthase and lysophosphatidic acid acyltransferase were both upregulated in DTR but showed little change in DSR. Based on GO term analysis, numerous DEGs in both DTR and DSR were associated with response to water deprivation and response to abscisic acid (Supplementary Data Fig. 2). Among the genes associated with response to abscisic acid, those encoding protein phosphatase 2C, U-box domain, and low-temperature-induced 65 kDa protein OS were expressed at a higher level in DTR. Some genes encoding homeobox-leucine zipper proteins and protein phosphatase 2C were also involved in the response to water deprivation pathway. Nine genes were annotated as protein phosphatase 2C, and three of them were upregulated more in DTR than DSR. Moreover, genes encoding aquaporin PIP, Arabidopsis histidine kinase 2/3/4 (cytokinin receptor), and another unannotated gene were upregulated in DTR but downregulated in DSR, and these genes may have potential for the improvement of plant drought resistance.

KEGG pathway and GO term analysis results were similar for leaves, and many DEGs were also associated with photosynthesis and the cell wall. More DEGs in DSL were associated with the term chloroplast, whereas more DEGs in DTL were associated with the term extracellular region (Supplementary Data Table 4, Fig. 3).

Genes differentially expressed between the two varieties

To search for important drought-response genes, we identified DEGs that were unique to the roots or leaves of individual varieties. DT roots had a larger number of DEGs than DS roots. Among these, the number of DEGs in all KEGG pathways was greater in DTR than in DSR, with the exception of the signal sensing and transduction pathway. In contrast to DSR, DEGs in signal sensing and transduction were all downregulated in DTR (Supplementary Data Fig. 3). It is also interesting to note that the GO term “structural constituent of ribosome” showed the most significant change in DTR under drought. By comparison, the term signal transduction appeared more frequently in DSR (Supplementary Data Fig. 2).

When comparing leaves of the two varieties, two secondary metabolite pathways were significantly changed in DTL only: stilbenoid, diarylheptanoid and gingerol biosynthesis, and flavonoid biosynthesis (Supplementary Data Table 5). Genes in the stilbenoid, diarylheptanoid and gingerol biosynthesis pathway were downregulated. Only one gene in the flavonoid biosynthesis pathway was upregulated. There were more DEGs in DSL than in DTL, and the expression of genes involved in fatty acid degradation changed markedly (Supplementary Data Table 6). Consistent with the KEGG analysis, GO term analysis showed that DEGs were enriched in the term photosynthesis in both genotypes. DTL showed the greatest change in the term extracellular region, but DSL showed the greatest change in the

term chloroplast (Fig. 3). In DTL, the most significantly enriched pathway was S-methyl-5-thioribose kinase activity: four DEGs were involved in this pathway, and all were upregulated.

Genes that shape drought tolerance in alfalfa

To better explore drought tolerance genes, we assumed that the DT genotype, which demonstrated greater drought tolerance based on physiological analyses, exhibited a higher level of expression of certain genes than DS under drought conditions. We therefore considered genes upregulated in DT but downregulated in DS to be drought tolerance genes; such genes were most prevalent in roots. The $\log_2$(fold-change) values of genes with similar expression patterns were averaged, and genes were divided into six groups based on gene function: carbon metabolism, amino acid metabolite, lipid metabolite, genetic processing, reactive oxygen species (ROS) and secondary metabolism (Fig. 4). These results suggested that carbohydrate metabolism, amino acid metabolites, and secondary metabolism may play important roles in alfalfa drought stress resistance. In addition, to better understand the coordinated regulation of genes in all tissues, we mapped the drought tolerance gene network regulation (Fig. 5). The results showed that the DT genotype significantly accumulated genes involved in lignin biosynthesis and wax layer metabolism. To further confirm these findings, we measured the contents of amino acids, soluble sugars, peroxidase, and lignin in different tissues and genotypes. The soluble sugar contents increased after drought stress, and there was greater soluble sugar accumulation in DT than in DS (Fig. 6A), a trend consistent with the RNA-seq results. After drought, amino acid contents increased in DTR, DSR and DSL but decreased non-significantly in DTL, a result that did not correlate with the RNA-seq data (Fig. 6B). POD content was significantly lower in DTR, DSR and DSL after drought, consistent with the RNA-seq data, which showed that POD gene expression was higher in DTL than in other groups (Fig. 6C). DTR had a higher SOD content than the other groups (Fig. 6D). The content of acid-soluble lignin was decreased under drought in all groups but was higher in DT than in DS (Fig. 6E), consistent with the RNA-seq and qPCR data (Fig. 7). Changes in cuticular wax on the lower leaf epidermis were observed by scanning electron microscopy. The wax distribution in DS was relatively sparse compared with DT (Fig. 6F), a result that was also in accord with the RNA-seq and qPCR data. Taken together, these results suggest that the main causes of drought resistance in DT may be higher levels of soluble sugar, peroxidase, lignin, and waxes.

qRT–PCR validation of differentially expressed transcripts from RNA-seq

To verify the reliability of the RNA-seq data, the expression of 17 DEGs involved in carbon metabolism, amino acid metabolism, SOD, response to water, lignin, and wax synthesis were analyzed by qRT–PCR (Supplementary Table 7). A correlation coefficient analysis showed that their expression changes were highly correlated between the two analyses (Fig. 7), confirming the reliability of the RNA-seq data.

Discussion

Plants react to drought stress at multiple levels, including biochemical, physiological, and developmental. Drought induces the transcription of metabolic genes, which contribute to the production and accumulation of osmotic substances that help to retain water and cellular antioxidants that protect cells from stress-related reactive oxygen species[34, 35]. In the present study, transcriptome analysis showed marked changes in gene expression of roots and leaves of alfalfa seedlings after 4 d of severe drought stress. Leaves exhibited more transcriptional changes than roots in drought-sensitive alfalfa, whereas roots exhibited more transcriptional changes than leaves in drought-tolerant alfalfa.

Genes involved in photosynthesis and carbon metabolism

Drought stress reduces the photosynthetic rate of plants, changes the distribution and metabolism of plant carbon, and causes decreased energy consumption and yield[36]. Here, downregulated expression of photosynthesis-related genes in drought-stressed leaves may have been associated with decreased photosynthetic capacity under drought stress[37]. The high number of downregulated genes associated with photosynthesis may also indicate that oxidative stress was higher in DSL than in DTL. Fructose-1,6-bisphosphatase (FBA) is an important metabolic enzyme involved in glycolysis, gluconeogenesis, and the Calvin cycle[38], and 6-phosphofructokinase 1 is a novel regulator indispensable for early chloroplast development[39]. Overexpression of FBA1 can alter growth, photosynthesis, and stress responses in higher plants[40, 41]. These two genes were upregulated in DTL, suggesting that they may have the potential to improve drought tolerance.

Drought stress is known to alter the activities of carbon-metabolizing enzymes[42]. Increased levels of soluble sugar and starch under drought stress indicates they are a preferred mechanism for maintaining root growth and metabolic processes[43]. Raffinose synthase is one of the key enzymes for the biosynthesis of sucrose into oligosaccharides[44]. It has been reported that raffinose synthase plays an important role in the osmotic regulation of nut seedling roots under drought stress[45]. ABA can increase raffinose biosynthesis and promote desiccation tolerance[46]. The expression of a raffinose synthase gene was markedly increased in DSL but decreased in DTR. From these results, we hypothesize that leaves of drought-sensitive alfalfa may produce more raffinose to resist severe drought stress. Moreover, 1,4-α-glucan, a branching enzyme, plays an important role in the biosynthesis of branched polysaccharides, glycogen, and amylopectin in carbohydrate metabolism[47]. A gene encoding this enzyme was up-regulated in DTL, suggesting that 1,4-α-glucan may contribute to its improved drought tolerance. Aldehyde dehydrogenase (NAD^+) is an important enzyme whose overexpression can enhance drought and salt tolerance[48]. The upregulation of the corresponding genes in DT was consistent with its higher level of drought tolerance. Two genes encode β-D-fructofuranosidase, which promotes cell growth[49], and we speculate that it is also very important for alfalfa drought tolerance.

In addition, carbohydrates regulate drought stress. In our study, genes encoding fructokinase and trehalose 6-phosphate were upregulated in DT. Fructokinase plays an important role in plant tolerance to abiotic stresses, and it can promote the accumulation of osmoprotectants under drought[50]. Trehalose 6-phosphate phosphatase controls the biosynthesis of trehalose[51], which functions as an osmolyte,

carbon reserve, transport sugar, stress protectant, and signaling and homeostatic regulator of sucrose in plants[52].

Lignin is the main structural component of thickened secondary cell walls in almost all vascular plants[53], and it is very important for drought tolerance[54]. Ferulate-5-hydroxylase is responsible for the final hydroxylation of syringyl-type lignin precursors. Laccase catalyzes the oxidation of various substrates while reducing molecular oxygen to water[55]and participates in lignin biosynthesis[56]. All of these genes were increased in DT, suggesting that increased lignin metabolism improves drought-tolerance.

Multiple cellulose-related genes were also upregulated. Endoglucanase is the main enzyme involved in the hydrolysis of biomass; it initiates cellulose hydrolysis and increases the cellulose hydrolysis rate. Ten genes encoded β-glucosidase, which hydrolyzes cellobiose into glucose[57, 58] and is the rate-limiting component of the cellulase-hydrolyzing reaction[59]. The biosynthesis of cellulose can be promoted by sucrose synthase[60], and three genes encoded sucrose synthase. Xylan is the main component of hemicellulose[61], and application of β-xylosidase together with xylanase improves xylan hydrolysis[62]. Together, we suggest that the cellulose-related genes may positively regulated the drought stress response in DT genotype.

Genes involved in hormone metabolism and root elongation

Many drought-responsive genes from both genotypes were involved in the signal transduction of plant hormones, especially ABA. ABA plays an important role in plant adaptation to environmental stress such as drought, cold, and high salinity[63]. β-Glucosidase functions with inactive ABA to produce active ABA, thereby increasing ABA levels in plants[64] and improving drought tolerance[64]. Six β-glucosidase genes were up-regulated in DTR only, indicating that these related genes may serve as candidate gene for alfalfa drought tolerance.

The root system is the first site of drought perception, and this environmental perception is reflected in changes in gene expression[65]. Ethylene can inhibit root elongation and promote the formation and elongation of root hairs[66]. There is increasing evidence that light and ethylene signaling pathways interact with auxin and other plant hormone signaling pathways[67, 68]. This system is usually composed of two proteins, a histidine kinase and a response regulator[69]. Interestingly, the expression of genes encoding Arabidopsis histidine kinase (HKs) and the transcription factor KUA1 OS were increased in DTR only, indicating that HKs may improve drought stress. HKs forms part of the multistep His–Asp phospho-relay that controls CK signaling in Arabidopsis[70]. HKs are important receptors widely involved in cellular reactions of bacteria, fungi, and plants[71]. In addition, HK can regulate root elongation by an ETR1-dependent abscisic acid signaling pathway. *Arabidopsis* HK 2/3/4 is a type of cytokinin receptor. Thus, we speculate that HK may have improved drought stress tolerance by promoting elongation of alfalfa roots. However, further studies are needed to determine the role of KUA1 OS in drought stress. In previous studies, drought stress inhibited the synthesis and transport of CK, decreasing the CK concentration and accelerating leaf senescence[72]. The exogenous application or endogenous increase

of CK content can significantly delay leaf senescence[73]. Therefore, upregulation of CK-related genes in roots may improve drought survival. Another ethylene-responsive element binding protein is EREBP, which belongs to a large transcription factor family that is unique and widely distributed in plants[74]. EREBP plays important roles in abiotic stress responses[75]. Increased EREBP expression in DTR may indicate increased drought tolerance. It is interesting that there was greater upregulation of histidine- and ethylene-related genes in DTR than in DSR, perhaps indicating that such genes promote root elongation in drought-tolerant alfalfa. Aquaporins (AQPs) are intrinsic plant proteins[76]that form channels in the plasma membrane and intracellular membranes, facilitating the passive movement of water among compartments[77]. In maize, an aquaporin gene has been shown to be involved in root elongation and stomatal movement[78]. Our results suggest that the aquaporin PIP may improve alfalfa drought tolerance. In summary, DT showed greater expression of root extension-related genes, which may have contributed to its greater drought tolerance.

Genes involved in ROS and the stability of the plant cell membrane

It is well known that drought stress increases the production of reactive oxygen species (ROS) in intercellular compartments such as chloroplasts, peroxisomes, and mitochondria[79]. SOS2 is the central signaling element of the SOS pathway and represents a large family of protein kinases with catalytic domains similar to that of yeast sucrose nonfermenting 1 (SNF1) and mammalian amp activated kinase (AMPK)[80]. Therefore, upregulation of genes responsible for AMPK synthesis in roots may result in better drought survival. Peroxidase plays an important role in plant stress responses by efficiently scavenging H_2O_2 in the cytosol and chloroplasts[81]. Polyamine-related genes were upregulated only in DTR and have been shown to inhibit lipoxygenase activity in lentils[82]. In addition, 4-hydroxyphenylacetaldehyde can be produced by the activity of primary-amine oxidase[83], an enzyme involved in the biosynthesis of various secondary metabolites and compounds including plastoquinone[84, 85]. Primary-amine oxidase is also widely regarded as a drug target for the treatment of inflammatory, vascular, and neurodegenerative diseases[86]. Therefore, an increased abundance of these genes may improve drought tolerance.

The stability of plant cell membranes and ionic stability are also very important under drought conditions. Lysophosphatidic acid acyltransferases are known to function in the new glycerolipid biosynthesis pathway (Kennedy pathway), using lysophosphatidic acid (LPA) and acyl coenzyme a to form phosphatidic acid (PA)[87]. Hence, greater amounts of lysophosphatidic acid acyltransferase promote greater plant cell membrane stability. On this basis, alfalfa with more 3-ketoacyl-CoA synthase (KTCS) and lysophosphatidic acid acyltransferase should exhibit greater drought tolerance.

Drought stress can lead to insufficient supply of essential elements, thereby impairing plant metabolism and function[88]. H^+-ATPase is an important transporter for the maintenance of membrane potential and the regulation of K^+ transmembrane gradients in mesophyll cells. Early activation of root hair cell PM H^+-ATPase triggers may increase the biosynthesis of major osmolytes, leading to upregulation of the water maintenance system[89]. It has been reported that maintenance of mesophyll K^+ in tea plants under

drought and rehydration is associated with regulation of plasma membrane H^+-ATPase activity. In tea mesophyll cells, drought stress inhibited plasma membrane H^+-ATPase activity, induced net H^+ inflow, and led to membrane potential depolarization, resulting in a large K^+ outflow[90]. Under drought conditions, the diffusion of potassium ions from soil to plant roots is impaired[91]. Therefore, maintaining adequate cellular K^+ has a positive effect on drought resistance of plants under water limitation[92]. The increased expression of genes encoding H^+-ATPases observed here may have therefore been helpful in reducing K^+ outflow under drought conditions and may have played a role in the improved drought tolerance of DTR. Ca^{2+} content normally increases in plants under abiotic stress[93]. In our results, Ca^{2+} accumulated in DT, especially in the roots, but decreased in the drought-sensitive cultivar. This result suggests that concentrations of Ca^{2+} may improve drought stress tolerance, but severe drought stress may cause decreased Ca^{2+} levels, and this hypothesis requires further study.

Genes involved insecondarymetabolism

Secondary metabolites play an important role in plant drought tolerance[94], and ubiquitination-mediated protein degradation can improve drought tolerance[95]. Speckle-type POZ protein is a key adaptor molecule of ubiquitination[96]and can promote ubiquitination[97]. Our results therefore suggest that speckle-type POZ protein may improve drought tolerance by promoting ubiquitination. RING-box protein 1 (RBX1) regulates physiological cellular functions in animal cells[98]and plays a key role in organismal development[99]. RING-box proteins contain a RING finger domain; as the catalytic components of cullin E3 ligases, they participate in the ubiquitination of target proteins for subsequent proteasome-dependent degradation[12]. E3 plays a unique role in the recognition of ubiquitinated target proteins[100]. Some E3 ubiquitin-protein ligase-like F-box proteins have important roles in drought stress[101]. RING-box protein 1 was upregulated in DT under drought stress, which may indicate that it has a role in the response of alfalfa to drought stress.

Cuticular wax helps limit non-stomatal transpiration and has been used as a marker in breeding and selection of drought-resistant wheat cultivars[102]. Alcohol-forming fatty acyl reductases produce fatty alcohols that have a single hydroxyl group at the terminal position; these are often components of plant extracellular lipid barriers like cell walls or cuticular waxes[103]. Four fatty acid omega-hydroxylase genes were upregulated in DT; their products are known as signaling molecules that act as mediators of plant defense reactions[104]. In plants, fatty acid omega-hydroxylase is essential for the synthesis of the cuticle[105], and fatty acids have also been found to improve drought tolerance[106]. 3-ketoacyl-CoA synthase encodes the first component of the fatty acid elongation complex[107] that is responsible for the synthesis of wax precursors and is therefore involved in limiting non-stomatal water loss and responding to drought stress[108]. Significantly upregulated wax metabolism genes may constitute additional candidate genes for drought tolerance.

In addition to the genes above, we also identified additional drought-related genes in DT. Branched chain amino acid aminotransferases (BCATs) specialize in the degradation of the l-branched chain amino acids

(BCAAs) leucine, isoleucine and valine using 2-oxoglutarate as an amino acceptor[109]. They have been reported to increase growth[110]. Chalcone synthase (CHS) catalyzes the first committed step in the biosynthesis of flavonoids[111], important secondary metabolites that play a key role in many aspects of plant growth and development[112]. Homogentisate phytyltransferase (HPT) is an important enzyme in the biosynthesis of vitamin E[113]. Vitamin E appears to be essential for plant development and participates in the response to a number of environmental stresses[114]. Three genes encoding tyrosine aminotransferase were upregulated in DTL. L-tyrosine is required for protein synthesis and also serves as a precursor for several plant metabolites, including alkaloids, phenylquinones, and cyanoglycosides[115]. Tyrosine aminotransferase (TAT) is the first key enzyme for the synthesis of important secondary metabolites [116] and has a proposed role in abiotic stress response[117]. It catalyzes the reversible conversion of tyrosine and 4-hydroxyphenylpyruvate in the tyrosine-derived pathway. Our results suggest that TAT may help to improve drought tolerance. In addition, two tropinone reductase (TR) genes were differentially expressedsmall proteins that belong to the short chain dehydrogenase/reductase family[118]. Their role in drought stress has received little attention and requires further study.

Genes involved in genetic processing

It is interesting that some genes involved in cell division were differentially expressed only in DT. NF-kappa B is a nuclear factor-κB (NF-κB) that participates in the regulation of inflammatory enzymes and cytokines[119]. According to our results, expression of the NF-kappa B gene may also improve drought stress tolerance. Three DEGs encoded origin recognition complex subunit 1 (ORC1), which is reported to be closely associated with the cell cycle[120] and is essential for the initiation of DNA replication[121]. Mitotic-specific cyclin-B1 and NADPH can activate CDK1 throughout early mitosis[122], and CDK1 can ensure repression of macroautophagy during mitosis[123]. Decreased expression of these genes in DT may have been associated with reduced macroautophagy in DTR. Carbon degradation repression (Crc) can directly inhibit the translation of mRNAs that encode enzymes. It can also indirectly inhibit the entry into cells of enzymes and transporters required for the translation of mRNAs encoding transcriptional regulators that drive the expression of genes whose products decompose specific substrates[124]. Dmc1 acts synergistically with the recombinase Rdh54 to mediate inter-homologue recombination during meiosis[125], and Dmc1 gene sequences are useful for resolution of the molecular phylogenetic relationships of tetraploid wheat[126]. We found few reports about the role of these cell cycle genes in drought tolerance, and additional research is needed to clarify their roles in alfalfa’s drought response.

Conclusions

Alfalfa has great economic and ecological importance, and drought stress is one of the major limitations on its growth and geographic distribution. Here, phenotypic, physiological, and transcriptomic analyses suggested that high contents of soluble sugars, lignin and wax were associated with greater drought stress tolerance in alfalfa. A number of upregulated genes appeared to have potential roles in drought tolerance, including those associated with ABA metabolism, root elongation, peroxidase activity, cell membrane stability, ubiquitination, and genetic processing. However, the drought-tolerance effects of

these candidate genes require further empirical confirmation. The results obtained here provide new insight into regulatory pathways of drought resistance, and functional analysis of these drought tolerance-related genes is an important avenue for future research.

Material And Methods

Plant materials and drought treatments

Two genotype alfalfa cloned populations, the drought-tolerant DT cultivar and the drought-sensitive DS cultivar, were selected for use in this study based on results from preliminary experiments. All plant materials were selected and cloned by cutting to ensure genotype consistency. The plants were transferred to plastic culture pots (10 cm height, 8 cm diameter, one plant per pot) that contained perlite and vermiculite (v/v, 1:1), with 18 pots per cultivar. The propagated plants grew in a greenhouse at 22°C and 40% relative humidity, and all plants were cut twice to ensure that their sizes were uniform. After 4 weeks, the plants were randomly assigned to control or drought stress groups. The control pots were continuously watered to maintain 100% field soil water capacity, whereas the drought pots were not continuously watered. The soil moisture level of each pot was measured in the morning and afternoon using a ProCheck with GS3 sensor. When the soil moisture level was lower than average and the variation was greater than 10%, we adjusted the soil moisture level to the average level by watering. Therefore, the soil moisture level of all pots was uniform, and all plants were exposed to similar drought stress conditions. Stress conditions were maintained until plants showed visible signs of wilting.

Phenotypic and biochemical analysis

Measurements of stem elongation rate and chlorophyll content were taken each morning at 9:00 AM for 6 d from the initiation to the cessation of drought stress. The stem length was measured each morning using a ruler, and the stem elongation rate was calculated as previously described[27]. The chlorophyll content was measured using a SPAD-502 meter (Konica Minolta, Osaka, Japan). Root length and all other physiological and biochemical parameters were measured after 4 d of drought stress. The root length was measured using a ruler. Leaf and root specimens were collected for biochemical measurements and transcriptome sequencing, and each replicate contained all leaves from an individual plant. The roots were dug out and washed free of soil under a stream of cold water. Six individual plants from each replicate of each treatment and cultivar were used, and the experiment was repeated three times. All specimens were collected between 10:00 AM and 12:00 PM and immediately frozen in liquid nitrogen. The concentrations of superoxide dismutase (SOD) and peroxidase (POD) were determined as previously described[28]. The content of free amino acids was determined by the ninhydrin method[29]. The sugar analysis was performed as previously described[30]with minor modifications. In brief, leaves and roots (0.1 g) were homogenized and transferred into test tubes to which 1 mL of 23% phenol solution was added. After incubation in a boiling water bath for 30 min, the supernatants were collected, and the absorbance was measured by UV spectrophotometry at 540 nm (Shimadzu, Japan). The concentrations

of K^+ and Ca^{2+} in leaves were determined by flame spectrophotometry (ColeParmer Instrument Co, Vernon Hills, IL, USA) as previously described[31].

The lignin concentration was measured as previously described [17] with minor modifications. In brief, dry specimens (100 mg) were ground, the cell wall materials were isolated using 0.01% sodium azide, and cell wall starch was digested with amylase and pullulans. The lignin level was quantified using the acetyl bromide method with an absorbance of 280 nm. An extinction coefficient of 15.693 was used to calculate the lignin concentration. The leaf wax content was determined by ESEM imaging. The middle sections of the second true leaves of alfalfa seedlings were randomly selected from control and treatment groups. The leaves were minced into fragments of 5 mm^2 and immediately fixed in 3% glutaric dialdehyde. The dehydrated samples were coated with gold particles using a sputtering machine in a vacuum dryer (Bal-Tec GmbH, Germany), and the epidermis was observed by environmental scanning electron microscopy (ESEM, Quanta-200, Fei Co., USA).

RNA sequencing and data analysis

Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. RNA quality and quantity were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Transcriptome sequencing and analysis were performed by OE Biotech Co., Ltd. (Shanghai, China). Raw reads were processed using Trimmomatic[32]. Both a *P*-value <0.05 and a fold-change >2 were used as the threshold to identify genes with significantly different levels of expression. Hierarchical clustering analysis of DEGs was performed to explore gene expression patterns. Gene Ontology (GO) and KEGG pathway enrichment analyses of all DEGs detected during different periods of drought were performed in R based on the hypergeometric distribution. The reads were reassembled using StringTie [33].

Validation of DEGs by qRT–PCR

To validate the RNA-seq data, 16 significant DEGs were selected for validation by qRT–PCR. The treatments and RNA isolation steps were performed as previously described. The primers for these genes were designed using Primer-BLAST at NCBI (Supplementary Table 1). We selected the actin gene as the endogenous control. The reverse-transcription reactions were performed using the PrimeScript Reagent Kit with gDNA Eraser (Takara, USA). Quantitative real time PCR was performed using TB Green Premix Ex Taq II (Takara). All analyses were performed using three biological replicates.

Declarations

Conflicts of interest

The authors declare no conflicts of interest.

Authors' contributions

QM Conceptualization, Validation, Resources, Writing: original draft, Writing: review & editing, Visualization. XX Validation, Visualization, Supervision. YX Visualization, Supervision. LZ performed the experiments, Software. WW analyzed the data, Formal analysis. DM: Validation, Visualization, Supervision, Project administration, Funding acquisition. All authors read

and approved the final manuscript.

Funding

This research was supported by the Project of First-Class Discipline Construction (Ecology) for Ningxia Higher Education (NXYLXK2017B06), National Natural Science Foundation of China (31760698) and Ningxia Agricultural Breeding Project 2019 (BBF02022-04), the Project of First-Class University of Western China for Key Laboratory of Ningxia University (GZXM2017001).

Acknowledgements

We also thank Prof. Xiqing Ma (China Agricultural University, College of Grass Science) for editorial suggestions.

References

1. Qiu Z, Yuan M, He Y, Li Y, Zhang LJSr. Physiological and transcriptome analysis of He-Ne laser pretreated wheat seedlings in response to drought stress. Scientific reports.2017; 7(1):1-12.
2. Han Y, Yang H, Wu M, Yi HJE, safety e. Enhanced drought tolerance of foxtail millet seedlings by sulfur dioxide fumigation. Ecotoxicology and Environmental Safety.2019; 178:9-16.
3. Xie X, Li A, Tan J, Lei G, Jin H, Zhang ZJEI. Uncertainty analysis of multiple global GPP datasets in characterizing the lagged effect of drought on photosynthesis. Ecological Indicators.2020; 113:106224.
4. Zhang C, Li X, He Y, Zhang J, Yan T, Liu XJPP, Biochemistry. Physiological investigation of C4-phosphoenolpyruvate-carboxylase-introduced rice line shows that sucrose metabolism is involved in the improved drought tolerance. Plant Physiology and Biochemistry.2017; 115:328-342.
5. Ahmed IM, Nadira UA, Bibi N, Cao F, He X, Zhang G, Wu FJE, Botany E. Secondary metabolism and antioxidants are involved in the tolerance to drought and salinity, separately and combined, in Tibetan wild barley. Environmental & Experimental Botany.2015; 111:1-12.
6. Neumann PMJAoB. Coping mechanisms for crop plants in drought-prone environments. Ann Bot.2008; 101(7):901-907.
7. Gowda VR, Henry A, Yamauchi A, Shashidhar H, Serraj RJFCR. Root biology and genetic improvement for drought avoidance in rice. Field Crops Research.2011; 122(1):1-13.
8. Deikman J, Petracek M, Heard JE. Drought tolerance through biotechnology: improving translation from the laboratory to farmers' fields. Curr Opin Biotechnol.2012; 23(2):243-250.

9. Jin H, Sun Y, Yang Q, Chao Y, Kang J, Jin H, Li Y, Margaret GJMbr. Screening of genes induced by salt stress from Alfalfa. Molecular biology reports.2010; 37(2):745-753.
10. Bouton JJE. The economic benefits of forage improvement in the United States. Euphytica 2007; 154(3):263-270.
11. Crochemore M-L, Huyghe C, Écalle C, Julier BJA. Structuration of alfalfa genetic diversity using agronomic and morphological characteristics. Relationships with RAPD markers. Agronomie.1998; 18(1):79-94.
12. Zhang C, Shi S, Liu Z, Yang F, Yin GJJoPP. Drought tolerance in alfalfa (*Medicago sativa* L.) varieties is associated with enhanced antioxidative protection and declined lipid peroxidation. Journal of Plant Physiology.2019; 232:226-240.
13. Fang Y, Xiong L. General mechanisms of drought response and their application in drought resistance improvement in plants. Cellular and molecular life sciences.2015; 72(4):673-689.
14. Zhao Y, Ma W, Wei X, Long Y, Zhao Y, Su M, Luo Q. Identification of Exogenous Nitric Oxide-Responsive miRNAs from Alfalfa (*Medicago sativa* L.) under Drought Stress by High-Throughput Sequencing. Genes.2020; 11(1):30.
15. Nuccio ML, Paul M, Bate NJ, Cohn J, Cutler SRJPe. Where are the drought tolerant crops? An assessment of more than two decades of plant biotechnology effort in crop improvement. Plant Science.2018(273):110-119.
16. Bao A-K, Wang S-M, Wu G-Q, Xi J-J, Zhang J-L, Wang C-M. Overexpression of the Arabidopsis H+-PPase enhanced resistance to salt and drought stress in transgenic alfalfa (*Medicago sativa* L.). Plant Science.2009; 176(2):232-240.
17. Tong Z, Li H, Zhang R, Ma L, Dong J, Wang T. Co-downregulation of the hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase and coumarate 3-hydroxylase significantly increases cellulose content in transgenic alfalfa (*Medicago sativa* L.). Plant Science.2015; 239:230-237.
18. Matthews C, Arshad M, Hannoufa A. Alfalfa response to heat stress is modulated by microRNA156. Physiologia Plantarum.2018; 165(4).
19. Krugman T, Peleg Z, Quansah L, Chagué V, Korol AB, Nevo E, Saranga Y, Fait A, Chalhoub B, Fahima T. Alteration in expression of hormone-related genes in wild emmer wheat roots associated with drought adaptation mechanisms. Funct Integr Genomics.2011; 11(4):565-583.
20. Moumeni A, Satoh K, Kondoh H, Asano T, Hosaka A, Venuprasad R, Serraj R, Kumar A, Leung H, Kikuchi S. Comparative analysis of root transcriptome profiles of two pairs of drought-tolerant and susceptible rice near-isogenic lines under different drought stress. BMC plant biology.2011; 11(1):1-17.
21. Opitz N, Paschold A, Marcon C, Malik WA, Lanz C, Piepho H-P, Hochholdinger F. Transcriptomic complexity in young maize primary roots in response to low water potentials. BMC genomics.2014; 15(1):741.
22. Du H, Shi Y, Li D, Fan W, Wang G, Wang CJPo. Screening and identification of key genes regulating fall dormancy in alfalfa leaves. PloS one.2017; 12(12):e0188964.

23. Lei Y, Xu Y, Hettenhausen C, Lu C, Shen G, Zhang C, Li J, Song J, Lin H, Wu JJBpb. Comparative analysis of alfalfa (*Medicago sativa* L.) leaf transcriptomes reveals genotype-specific salt tolerance mechanisms. BMC plant biology.2018; 18(1):1-14.
24. Merchan F, Lorenzo Ld, Rizzo SG, Niebel A, Manyani H, Frugier F, Sousa C, Crespi MJTPJ. Identification of regulatory pathways involved in the reacquisition of root growth after salt stress in Medicago truncatula. The Plant Journal.2007; 51(1):1-17.
25. Postnikova OA, Shao J, Nemchinov LGJP. Analysis of the alfalfa root transcriptome in response to salinity stress. Plant cell physiology.2013; 54(7):1041-1055.
26. Zhu H, Choi H-K, Cook DR, Shoemaker RCJPp. Bridging model and crop legumes through comparative genomics. 2005; 137(4):1189-1196.
27. Ballaré C, Scopel A, Sánchez R. Photocontrol of stem elongation in plant neighbourhoods: effects of photon fluence rate under natural conditions of radiation. Plant Cell and Environment.1991; 14(1):57-65.
28. Meloni DA, Oliva MA, Martinez CA, Cambraia JJE. Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environmental Experimental Botany.2003; 49(1):69-76.
29. Chen Y, Fu X, Mei X, Zhou Y, Cheng S, Zeng L, Dong F, Yang Z. Proteolysis of chloroplast proteins is responsible for accumulation of free amino acids in dark-treated tea (*Camellia sinensis*) leaves. Journal of proteomics.2017; 157:10-17.
30. Liu H, Fu Y, Hu D, Yu J, Liu HJEH. Effect of green, yellow and purple radiation on biomass, photosynthesis, morphology and soluble sugar content of leafy lettuce via spectral wavebands “knock out”. Scientia Horticulturae.2018; 236:10-17.
31. Patakas A, Nikolaou N, Zioziou E, Radoglou K, Noitsakis BJPs. The role of organic solute and ion accumulation in osmotic adjustment in drought-stressed grapevines. Plant science.2002; 163(2):361-367.
32. Bolger AM, Lohse M, Usadel BJB. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics.2014; 30(15):2114-2120.
33. Pertea M, Pertea GM, Antonescu CM, Chang T-C, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nature biotechnology.2015; 33(3):290-295.
34. Chen Z, Zhu D, Wu J, Cheng Z, Yan X, Deng X, Yan YJSR. Identification of differentially accumulated proteins involved in regulating independent and combined osmosis and cadmium stress response in Brachypodium seedling roots. Scientific reports.2018; 8(1):1-17.
35. Hayano-Kanashiro C, Calderón-Vázquez C, Ibarra-Laclette E, Herrera-Estrella L, Simpson JJPo. Analysis of gene expression and physiological responses in three Mexican maize landraces under drought stress and recovery irrigation. PloS one.2009; 4(10):e7531.
36. CUELLAR-ORTIZ SM, DE LA PAZ ARRIETA-MONTIEL M, ACOSTA-GALLEGOS J, Covarrubias AAJP, cell. Relationship between carbohydrate partitioning and drought resistance in common bean. Plant, cell environmental & Experimental Botany.2008; 31(10):1399-1409.

37. Farkas D, Hansson ÖJFL. Thioredoxin-mediated reduction of the photosystem I subunit PsaF and activation through oxidation by the interaction partner plastocyanin. Febs Letters.2011; 585(12):1753-1758.
38. Patipong T, Ngoennet S, Honda M, Hibino T, Waditee-Sirisattha R, Kageyama H. A class I fructose-1, 6-bisphosphate aldolase is associated with salt stress tolerance in a halotolerant cyanobacterium Halothece sp. PCC 7418. Archives of biochemistry biophysics.2019; 672:108059.
39. Zhu X, Ze M, Yin J, Chern M, Wang M, Zhang X, Deng R, Li Y, Liao H, Wang L. A phosphofructokinase B-type carbohydrate kinase family protein, PFKB1, is essential for chloroplast development at early seedling stage in rice. Plant Science.2020; 290:110295.
40. Koo KM, Jung S, Kim J-B, Kim SH, Kwon SJ, Jeong W-J, Chung GH, Kang S-Y, Choi Y-E, Ahn J-W. Effect of ionizing radiation on the DNA damage response in Chlamydomonas reinhardtii. Genes Genomics.2017; 39(1):63-75.
41. Lu W, Tang X, Huo Y, Xu R, Qi S, Huang J, Zheng C, Wu C-aJG. Identification and characterization of fructose 1, 6-bisphosphate aldolase genes in Arabidopsis reveal a gene family with diverse responses to abiotic stresses. Gene.2012; 503(1):65-74.
42. Hou J, Huang X, Sun W, Du C, Wang C, Xie Y, Ma Y, Ma D. Accumulation of water-soluble carbohydrates and gene expression in wheat stems correlates with drought resistance. Journal of plant physiology.2018; 231:182-191.
43. Du Y, Zhao Q, Chen L, Yao X, Zhang W, Zhang B, Xie FJPP. Effect of drought stress on sugar metabolism in leaves and roots of soybean seedlings. Plant Physiology Biochemistry.2020; 146:1-12.
44. Sui X-l, Meng F-z, Wang H-y, Wei Y-x, Li R-f, Wang Z-y, Hu L-p, Wang S-h, Zhang Z-xJJopp. Molecular cloning, characteristics and low temperature response of raffinose synthase gene in Cucumis sativus L. Journal of plant physiology.2012; 169(18):1883-1891.
45. Zhang C, Zhang L, Zhang S, Zhu S, Wu P, Chen Y, Li M, Jiang H, Wu GJBPB. Global analysis of gene expression profiles in physic nut (*Jatropha curcas* L.) seedlings exposed to drought stress. BMC plant biology.2015; 15(1):1-14.
46. Zhang Y, Sun Q, Zhang C, Hao G, Wang C, Dirk LM, Downie AB, Zhao T. Maize VIVIPAROUS1 interacts with ABA INSENSITIVE5 to regulate GALACTINOL SYNTHASE2 expression controlling seed raffinose accumulation. Journal of agricultural food chemistry.2019; 67(15):4214-4223.
47. Li D, Fei T, Wang Y, Zhao Y, Dai L, Fu X, Li X. A cold-active 1, 4-α-glucan branching enzyme from Bifidobacterium longum reduces the retrogradation and enhances the slow digestibility of wheat starch. Food Chemistry.2020:126855.
48. Yang H, Zhang D, Li H, Dong L, Lan H. Ectopic overexpression of the aldehyde dehydrogenase ALDH21 from Syntrichia caninervis in tobacco confers salt and drought stress tolerance. Plant Physiology Biochemistry.2015; 95:83-91.
49. Chi Z, Liu N-N, Jiang H, Wang Q-Q, Chen J-T, Liu G-L, Hu Z, Chi Z-MJIjobm. Relationship between β-D-fructofuranosidase activity, fructooligosaccharides and pullulan biosynthesis in Aureobasidium melanogenum P16. International journal of biological macromolecules.2019; 125:1103-1111.

50. Yang J, Zhan R, Jin Y, Song J, Li D, An G, Li M. Functional analysis of the promoter of the MdFRK2 gene encoding a high-affinity fructokinase in apple (*malus× domestica*). Scientia Horticulturae.2020; 265:109088.
51. Qiu L, Wei X-Y, Wang S-J, Wang J-JJPB. Characterization of trehalose-6-phosphate phosphatase in trehalose biosynthesis, asexual development, stress resistance and virulence of an insect mycopathogen. Pesticide Biochemistry Physiology.2020; 163:185-192.
52. Baena-González E, Lunn JEJCOiPB. SnRK1 and trehalose 6-phosphate–two ancient pathways converge to regulate plant metabolism and growth. Current opinion in plant biology.2020; 55:52-59.
53. Sibout R, Baucher M, Gatineau M, Van Doorsselaere J, Mila I, Pollet B, Maba B, Pilate G, Lapierre C, Boerjan W. Expression of a poplar cDNA encoding a ferulate-5-hydroxylase/coniferaldehyde 5-hydroxylase increases S lignin deposition in Arabidopsis thaliana. Plant physiology biochemistry.2002; 40(12):1087-1096.
54. Mazloom N, Khorassani R, Zohury GH, Emami H, Whalen J. Lignin-based hydrogel alleviates drought stress in maize. Environmental Experimental Botany.2020:104055.
55. Xu X, Zhou Y, Wang B, Ding L, Wang Y, Luo L, Zhang Y, Kong WJG. Genome-wide identification and characterization of laccase gene family in Citrus sinensis. Gene.2019; 689:114-123.
56. Cho HY, Lee C, Hwang S-G, Park YC, Lim HL, Jang CSJG. Overexpression of the OsChI1 gene, encoding a putative laccase precursor, increases tolerance to drought and salinity stress in transgenic Arabidopsis. Gene.2014; 552(1):98-105.
57. Phadungcharoen N, Winotapun W, Khomniyawanit A, Krataichan F, Rojanarata T. Facile and green fabrication of biocatalytic chitosan beads by one-step genipin-mediated β-glucosidase immobilization for production of bioactive genistein. Sustainable Chemistry Pharmacy.2019; 14:100187.
58. Kumar N, Sudan SK, Garg R, Sahni GJPB. Enhanced production of novel halostable recombinant endoglucanase derived from the metagenomic library using fed-batch fermentation. Process Biochemistry.2019; 78:1-7.
59. Liu X, Cao L, Zeng J, Liu Y, Xie WJljobm. Improving the cellobiose-hydrolysis activity and glucose-tolerance of a thermostable β-glucosidase through rational design. International journal of biological macromolecules.2019; 136:1052-1059.
60. Backes A, Behr M, Xu X, Gatti E, Legay S, Predieri S, Hausman J-F, Deyholos MK, Cai G, Guerriero G. Sucrose synthase gene expression analysis in the fibre nettle (*Urtica dioica* L.) cultivar “clone 13”. Industrial crops products.2018; 123:315-322.
61. Jin X, Song J, Ma J, Liu G-Q. Thermostable β-xylosidase from Aspergillus fumigatus: Purification, characterization and potential application in lignocellulose bioethanol production. Renewable Energy.2020.
62. Li T, Wu Y-R, He J. Heterologous expression, characterization and application of a new β-xylosidase identified in solventogenic Clostridium sp. strain BOH3. Process Biochemistry.2018; 67:99-104.

63. Gong Z, Xiong L, Shi H, Yang S, Herrera-Estrella LR, Xu G, Chao D-Y, Li J, Wang P-Y, Qin F. Plant abiotic stress response and nutrient use efficiency. SCIENCE CHINA Life Sciences.2020; 63(5):635-674.
64. Dietz KJ, Sauter A, Wichert K, Messdaghi D, Hartung WJJoEB. Extracellular β-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. Journal of experimental botany.2000; 51(346):937-944.
65. Janiak A, Kwasniewski M, Sowa M, Gajek K, Żmuda K, Kościelniak J, Szarejko IJFiPS. No time to waste: Transcriptome study reveals that drought tolerance in barley may be attributed to stressed-like expression patterns that exist before the occurrence of stress. Frontiers in plant science.2018; 8:2212.
66. Lin R-C, Park H-J, Wang H-Y. Role of Arabidopsis RAP2. 4 in regulating light-and ethylene-mediated developmental processes and drought stress tolerance. Molecular Plant.2008; 1(1):42-57.
67. Poupin MJ, Greve M, Carmona V, Pinedo IJFips. A complex molecular interplay of auxin and ethylene signaling pathways is involved in Arabidopsis growth promotion by Burkholderia phytofirmans PsJN. Frontiers in plant science.2016; 7:492.
68. Zhao Q, Guo H-WJMP. Paradigms and paradox in the ethylene signaling pathway and interaction network. Molecular plant.2011; 4(4):626-634.
69. Pirrung MC. Histidine kinases and two-component signal transduction systems. Cell Chemical Biology.1999; 6(6):R167-R175.
70. Li W, Herrera-Estrella L, Tran L-SP. The Yin–Yang of cytokinin homeostasis and drought acclimation/adaptation. Trends in plant science.2016; 21(7):548-550.
71. Nataraja KN, Parvathi MS: Tolerance to drought stress in plants: unravelling the signaling networks. In: Drought Stress Tolerance in Plants. Springer; 2016: 71-90.
72. Kudoyarova GR, Vysotskaya LB, Cherkozyanova A, Dodd ICJJoeb. Effect of partial rootzone drying on the concentration of zeatin-type cytokinins in tomato (Solanum lycopersicum L.) xylem sap and leaves. Journal of experimental botany.2007; 58(2):161-168.
73. Ma X, Zhang J, Burgess P, Rossi S, Huang B. Interactive effects of melatonin and cytokinin on alleviating drought-induced leaf senescence in creeping bentgrass (*Agrostis stolonifera*). Environmental Experimental Botany.2018; 145:1-11.
74. Cao Y, Wu Y, Zheng Z, Song FJP. Overexpression of the rice EREBP-like gene OsBIERF3 enhances disease resistance and salt tolerance in transgenic tobacco. Physiological Molecular Plant Pathology.2005; 67(3-5):202-211.
75. Kizis D, Lumbreras V, Pagès MJFL. Role of AP2/EREBP transcription factors in gene regulation during abiotic stress. FEBS letters.2001; 498(2-3):187-189.
76. Hasan S, Rabei S, Nada R, Abogadallah GJBp. Water use efficiency in the drought-stressed sorghum and maize in relation to expression of aquaporin genes. Biologia plantarum.2017; 61(1):127-137.
77. Merlaen B, De Keyser E, Ding L, Leroux O, Chaumont F, Van Labeke M-C. Physiological responses and aquaporin expression upon drought and osmotic stress in a conservative vs prodigal Fragaria x ananassa cultivar. Plant Physiology Biochemistry.2019; 145:95-106.

78. Iwuala E, Odjegba V, Sharma V, Alam AJCPB. Drought stress modulates expression of aquaporin gene and photosynthetic efficiency in *Pennisetum glaucum* (L.) R. Br. genotypes. Current Plant Biology.2020; 21:100131.
79. Ishibashi Y, Yamaguchi H, Yuasa T, Iwaya-Inoue M, Arima S, Zheng S-H. Hydrogen peroxide spraying alleviates drought stress in soybean plants. Journal of plant physiology.2011; 168(13):1562-1567.
80. Zhu, Cell J-KJ. Abiotic Stress Signaling and Responses in Plants. Cell.2016; 167(2):313-324.
81. Csiszár J, Gallé Á, Horváth E, Dancsó P, Gombos M, Váry Z, Erdei L, Györgyey J, Tari I. Different peroxidase activities and expression of abiotic stress-related peroxidases in apical root segments of wheat genotypes with different drought stress tolerance under osmotic stress. Plant physiology.2012; 52:119-129.
82. Maccarrone M, Veldink GA, Vliegenthart JF, Finazzi Agrò AJFl. Ozone stress modulates amine oxidase and lipoxygenase expression in lentil (*Lens culinaris*) seedlings 1. FEBS letters.1997; 408(2):241-244.
83. Yu T, Yin Y, Ge Y, Cheng S, Zhang X, Feng Z, Zhang J. Enzymatic production of 4-hydroxyphenylacetaldehyde by oxidation of the amino group of tyramine with a recombinant primary amine oxidase. Process Biochemistry.2020.
84. Huang B, Yi B, Duan Y, Sun L, Yu X, Guo J, Chen WJMBR. Characterization and expression profiling of tyrosine aminotransferase gene from Salvia miltiorrhiza (Dan-shen) in rosmarinic acid biosynthesis pathway. Molecular biology reports.2008; 35(4):601-612.
85. Wang B, Sun W, Li Q, Li Y, Luo H, Song J, Sun C, Qian J, Zhu Y, Hayward AJP. Genome-wide identification of phenolic acid biosynthetic genes in *Salvia miltiorrhiza*. Planta.2015; 241(3):711-725.
86. Shanahan P, O'Sullivan J, Tipton KF, Kinsella G, Ryan BJ, Henehan GT. Dietary phytochemicals as inhibitors of primary amine oxidase. Journal of Biotechnology.2017(256):S96-S97.
87. Eto M, Shindou H, Shimizu T. A novel lysophosphatidic acid acyltransferase enzyme (LPAAT4) with a possible role for incorporating docosahexaenoic acid into brain glycerophospholipids. Biochemical biophysical research communications.2014; 443(2):718-724.
88. Cetinkaya H, Koc M, Kulak M. Monitoring of mineral and polyphenol content in olive leaves under drought conditions: Application chemometric techniques. Industrial Crops Products.2016; 88:78-84.
89. Gong D-S, Xiong Y-C, Ma B-L, Wang T-M, Ge J-P, Qin X-L, Li P-F, Kong H-Y. Early activation of plasma membrane H+-ATPase and its relation to drought adaptation in two contrasting oat (*Avena sativa* L.) genotypes. Environmental and Experimental Botany.2010; 69(1):1-8.
90. Zhang X, Wu H, Chen L, Liu L, Wan X. Maintenance of mesophyll potassium and regulation of plasma membrane H^+-ATPase are associated with physiological responses of tea plants to drought and subsequent rehydration. The Crop Journal.2018; 6(6):611-620.
91. Wang M, Zheng Q, Shen Q, Guo S. The critical role of potassium in plant stress response. International journal of molecular sciences.2013; 14(4):7370-7390.

92. Demidchik V, Cuin TA, Svistunenko D, Smith SJ, Miller AJ, Shabala S, Sokolik A, Yurin V. Arabidopsis root K^+-efflux conductance activated by hydroxyl radicals: single-channel properties, genetic basis and involvement in stress-induced cell death. Journal of cell science.2010; 123(9):1468-1479.
93. Kolupaev YE, Karpets YV, Dmitriev A. Signal mediators in plants in response to abiotic stress: calcium, reactive oxygen and nitrogen species. Cytology Genetics.2015; 49(5):338-348.
94. Gao S, Wang Y, Yu S, Huang Y, Liu H, Chen W, He X. Effects of drought stress on growth, physiology and secondary metabolites of Two Adonis species in Northeast China. Scientia Horticulturae.2020; 259:108795.
95. Zhang Z, Li J, Liu H, Chong K, Xu Y. Roles of ubiquitination-mediated protein degradation in plant responses to abiotic stresses. Environmental Experimental Botany.2015; 114:92-103.
96. Ji P, Liang S, Li P, Xie C, Li J, Zhang K, Zheng X, Feng M, Li Q, Jiao H. Speckle-type POZ protein suppresses hepatocellular carcinoma cell migration and invasion via ubiquitin-dependent proteolysis of SUMO1/sentrin specific peptidase 7. Biochemical biophysical research communications.2018; 502(1):30-42.
97. Gan W, Dai X, Lunardi A, Li Z, Inuzuka H, Liu P, Varmeh S, Zhang J, Cheng L, Sun Y. SPOP promotes ubiquitination and degradation of the ERG oncoprotein to suppress prostate cancer progression. Molecular cell.2015; 59(6):917-930.
98. Altintas E, Kaynar M, Celik ZE, Celik M, Kilic O, Akand M, Goktas S: Expression of Ring Box-1 protein and its relationship with Fuhrman grade and other clinical-pathological parameters in renal cell cancer. In: Urologic Oncology: Seminars and Original Investigations: 2020. Elsevier: 6. e17-16. e22.
99. Wang X, Wang X, Wang W, Zhang J, Wang J, Wang C, Lv M, Zuo T, Liu D, Zhang HJB. Both Rbx1 and Rbx2 exhibit a functional role in the HIV-1 Vif-Cullin5 E3 ligase complex in vitro. Biochemical Biophysical Research Communications.2015; 461(4):624-629.
100. Hellmann H, Estelle M. Plant development: regulation by protein degradation. Science.2002; 297(5582):793-797.
101. Gao S, Song JB, Wang Y, Yang ZMJE. An F-box E3 ubiquitin ligase-coding gene AtDIF1 is involved in Arabidopsis salt and drought stress responses in an abscisic acid-dependent manner. Environmental Experimental Botany.2017; 138:21-35.
102. Su R, Chen L, Wang Z, Hu Y. Differential response of cuticular wax and photosynthetic capacity by glaucous and non-glaucous wheat cultivars under mild and severe droughts. Plant physiology.2020; 147:303-312.
103. Rowland O, Domergue F. Plant fatty acyl reductases: enzymes generating fatty alcohols for protective layers with potential for industrial applications. Plant Science.2012; 193:28-38.
104. Benveniste I, Saito T, Wang Y, Kandel S, Huang H, Pinot F, Kahn RA, Salaün J-P, Shimoji M. Evolutionary relationship and substrate specificity of Arabidopsis thaliana fatty acid omega-hydroxylase. Plant science.2006; 170(2):326-338.
105. Heredia AJBEBA. Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer. Biochimica et Biophysica Acta -General Subjects.2003; 1620(1-3):1-7.

106. Bayati P, Karimmojeni H, Razmjoo J. Changes in essential oil yield and fatty acid contents in black cumin (*Nigella sativa* L.) genotypes in response to drought stress. Industrial Crops and Products.2020; 155:112764.
107. Fan Q, Creamer R, Li Y. Time-course metabolic profiling in alfalfa leaves under Phoma medicaginis infection. PloS one.2018; 13(10):e0206641.
108. Zhang G, Shan S, Wu Y, Huang S, Li D, Deng J, Qi J. The KCS gene is involved in the formation of chloroplast stromules and other physiological processes in jute (*Corchorus capsularis* L.). Industrial Crops Products.2019; 141:111781.
109. Stekhanova TN, Rakitin AL, Mardanov AV, Bezsudnova EY, Popov VO. A novel highly thermostable branched-chain amino acid aminotransferase from the crenarchaeon Vulcanisaeta moutnovskia. Enzyme microbial technology.2017; 96:127-134.
110. Campbell MA, Patel JK, Meyers JL, Myrick LC, Gustin JL. Genes encoding for branched-chain amino acid aminotransferase are differentially expressed in plants. Plant physiology Biochemistry.2001; 39(10):855-860.
111. Yahyaa M, Ali S, Davidovich-Rikanati R, Ibdah M, Shachtier A, Eyal Y, Lewinsohn E, Ibdah M. Characterization of three chalcone synthase-like genes from apple (*Malus x domestica Borkh*.). Phytochemistry.2017; 140:125-133.
112. Shuai C, ZHANG Y-c, PAN X-h, LI Y-t, CUI L-j, WU F-y, CAO M-j, YANG A-g, PAN G-tJJoia. Isolation and expression analysis of NtCHS6, a new chalcone synthase gene from Nicotiana tabacum. Journal of integrative agriculture.2017; 16(7):1443-1450.
113. Seo YS, Kim SJ, Harn CH, Kim WT. Ectopic expression of apple fruit homogentisate phytyltransferase gene (MdHPT1) increases tocopherol in transgenic tomato (Solanum lycopersicum cv. Micro-Tom) leaves and fruits. Phytochemistry.2011; 72(4-5):321-329.
114. Muñoz P, Munné-Bosch S. Vitamin E in Plants: Biosynthesis, Transport, and Function. Trends in plant science.2019; 24(11):1040-1051.
115. Wang M, Toda K, Maeda HAJP. Biochemical properties and subcellular localization of tyrosine aminotransferases in Arabidopsis thaliana. Hytochemistry.2016; 132:16-25.
116. Sobrado VR, Montemartini-Kalisz M, Kalisz HM, de la Fuente MC, Hecht HJ, Nowicki C. Involvement of conserved asparagine and arginine residues from the N-terminal region in the catalytic mechanism of rat liver and Trypanosoma cruzi tyrosine aminotransferases. Protein science.2003; 12(5):1039-1050.
117. Wang H, Dong Q, Duan D, Zhao S, Li M, van Nocker S, Ma F, Mao KJPP. Comprehensive genomic analysis of the TYROSINE AMINOTRANSFERASE (TAT) genes in apple (*Malus domestica*) allows the identification of MdTAT2 conferring tolerance to drought and osmotic stresses in plants. Plant Physiology Biochemistry.2018; 133:81-91.
118. Kushwaha AK, Sangwan NS, Tripathi S, Sangwan RSJG. Molecular cloning and catalytic characterization of a recombinant tropine biosynthetic tropinone reductase from Withania coagulans leaf. Gene.2013; 516(2):238-247.

119. Linghu K-G, Zhao GD, Xiong W, Sang W, Xiong SH, Tse AKW, Hu Y, Bian Z, Wang Y, Yu HJJoE. Comprehensive comparison on the anti-inflammatory effects of three species of Sigesbeckia plants based on NF-κB and MAPKs signal pathways in vitro. Journal of Ethnopharmacology.2020; 250:112530.
120. Xiong W, Xie C, Qiu Y, Tu Z, Gong Q. Origin recognition complex subunit 1 regulates cell growth and metastasis in glioma by altering activation of ERK and JNK signaling pathway. Molecular Cellular Probes.2020; 49:101496.
121. Mehra P, Biswas AK, Gupta A, Gourinath S, Chitnis CE, Dhar SK. Expression and characterization of human malaria parasite Plasmodium falciparum origin recognition complex subunit 1. Biochemical biophysical research communications.2005; 337(3):955-966.
122. Levasseur MD, Thomas C, Davies OR, Higgins JM, Madgwick S. Aneuploidy in oocytes is prevented by sustained CDK1 activity through degron masking in cyclin B1. Developmental cell.2019; 48(5):672-684. e675.
123. Odle RI, Walker SA, Oxley D, Kidger AM, Balmanno K, Gilley R, Okkenhaug H, Florey O, Ktistakis NT, Cook SJ. An mTORC1-to-CDK1 switch maintains autophagy suppression during mitosis. Molecular cell.2020; 77(2):228-240. e227.
124. Johnson CW, Abraham PE, Linger JG, Khanna P, Hettich RL, Beckham GT. Eliminating a global regulator of carbon catabolite repression enhances the conversion of aromatic lignin monomers to muconate in Pseudomonas putida KT2440. Metabolic engineering communications.2017; 5:19-25.
125. Chi P, Kwon Y, Moses DN, Seong C, Sehorn MG, Singh AK, Tsubouchi H, Greene EC, Klein HL, Sung P. Functional interactions of meiotic recombination factors Rdh54 and Dmc1. DNA repair.2009; 8(2):279-284.
126. Tang Y, Kang H-Y, Tang L, Diao C-D, Li D-Y, Zhu W, Fan X, Wang Y, Zeng J, Xu L-L. Phylogenetic analysis of tetraploid wheat based on nuclear DMC1 gene. Biochemical Systematics Ecology.2017; 70:239-246.

Figures

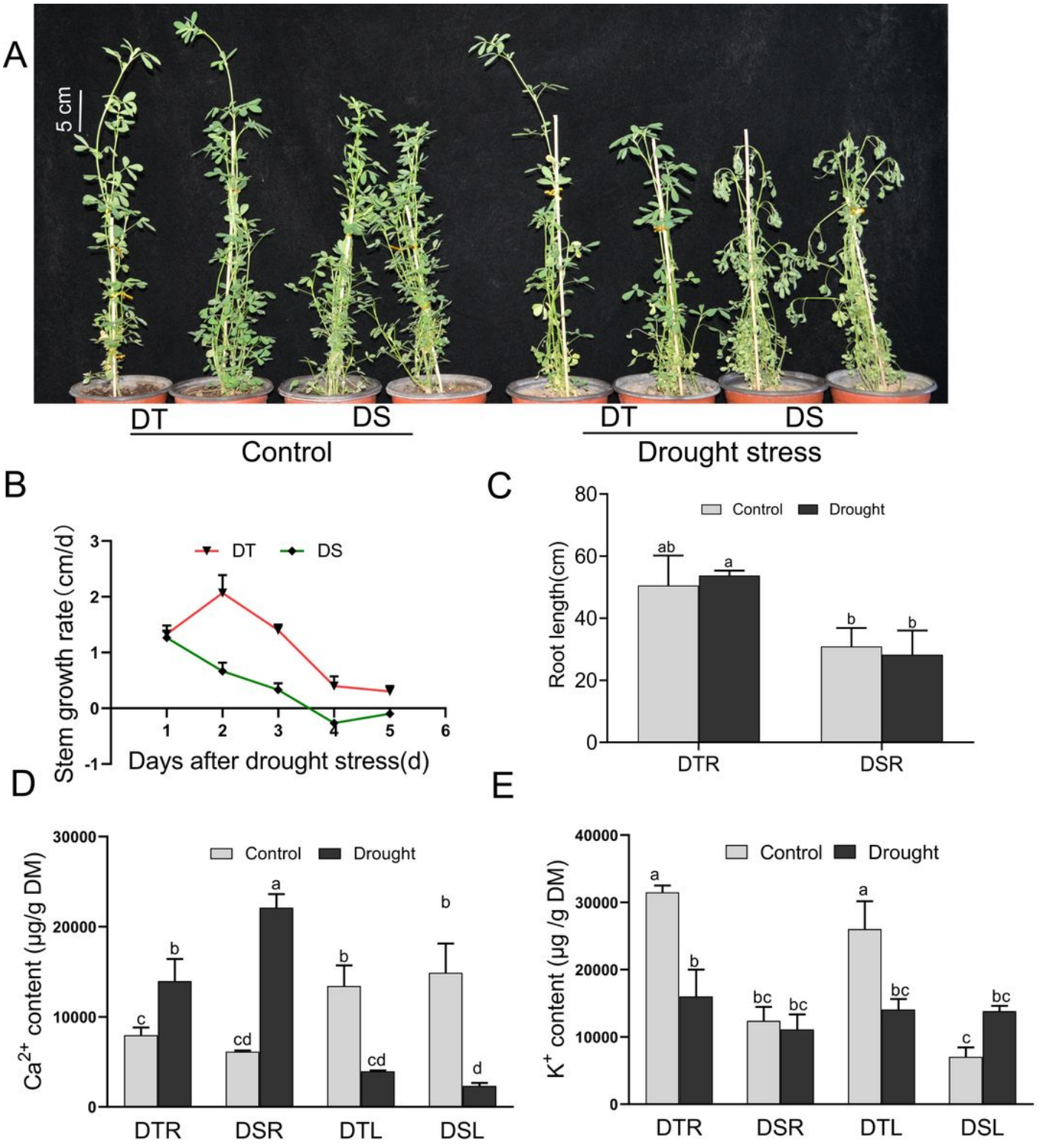

Figure 1

Drought responses of drought tolerant (DT) and drought sensitive (DS) alfalfa plants. (A) Phenotypes after 4 d of water restriction in the growth chamber. (B) Root length under control and drought stress conditions. (C) Stem growth rate over 4 d of drought stress. (D) Ca2+ content under control and drought stress conditions. (E) K+ content under control and drought stress conditions.

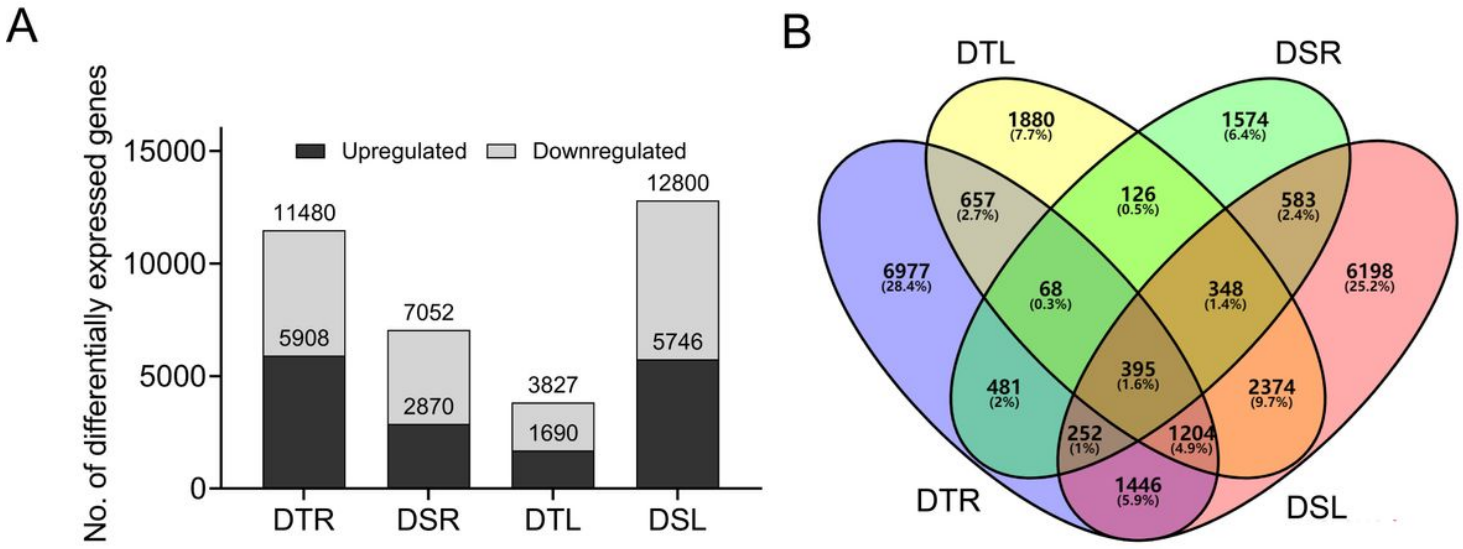

Figure 2

Genes differentially expressed in roots and leaves of two alfalfa cultivars under control and drought conditions. (A) Number of DEGs under control and drought conditions ($P < 0.05$, $FC \geq 2$). (B) Venn diagram of genes differentially expressed after drought stress in roots and leaves of two alfalfa cultivars.

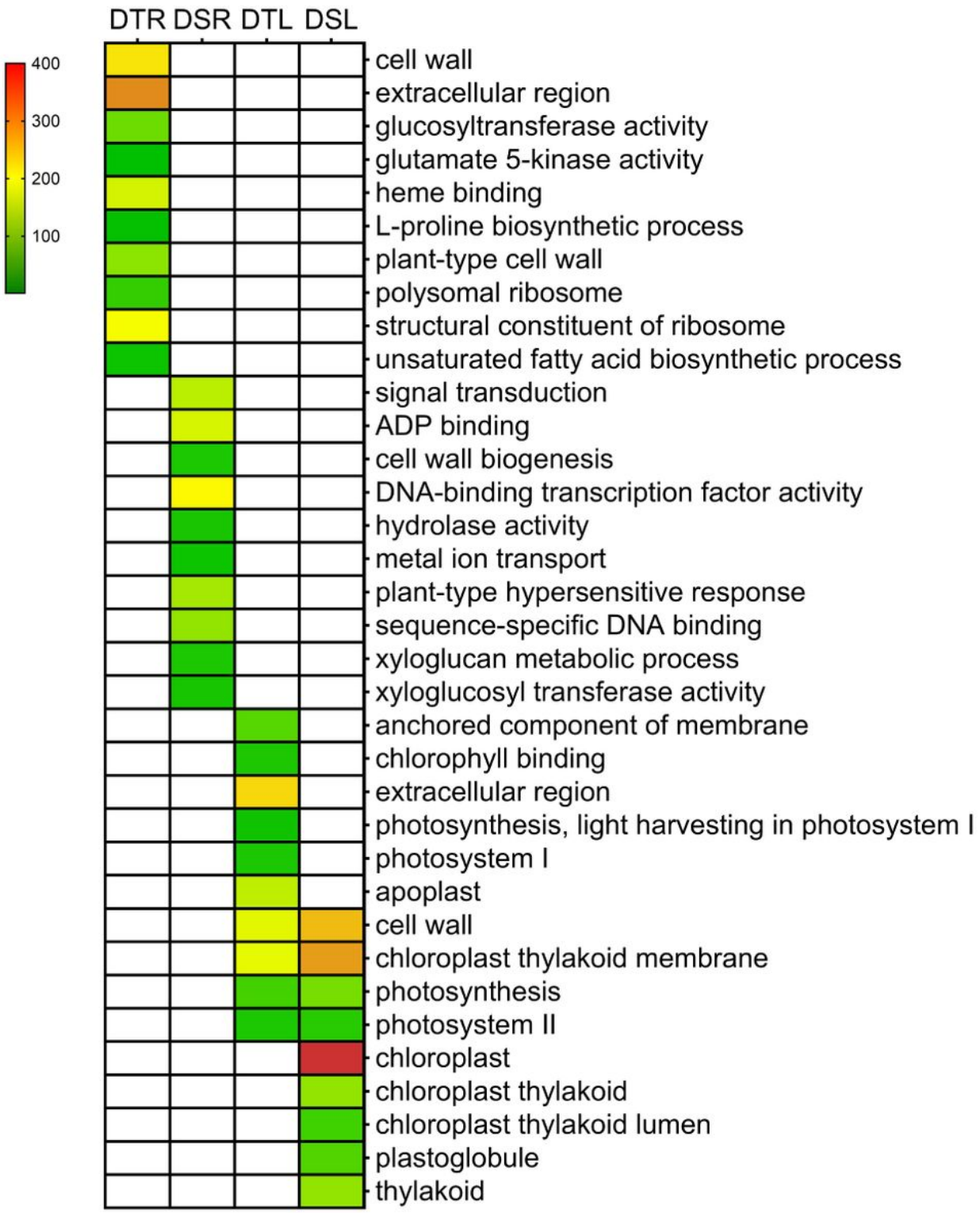

Figure 3

Top ten significantly (FDR < 0.05) associated GO terms for genes that were differentially expressed in roots or leaves of two alfalfa cultivars under drought.

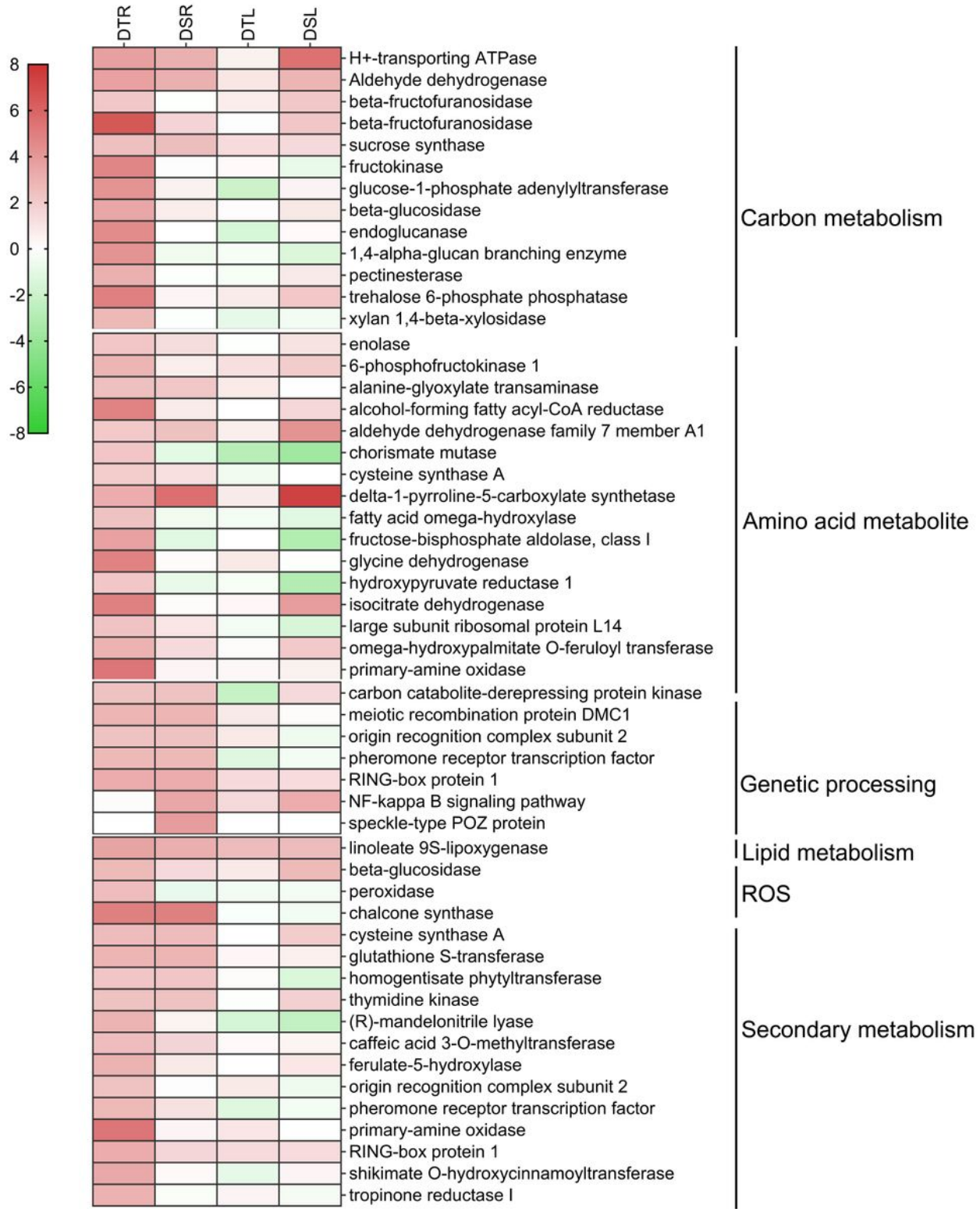

Figure 4

Heatmap expression profile of DEGs associated with drought tolerance. The colour scale indicates log2-transformed gene expression levels. Red indicates high transcript abundance, green indicates low transcript abundance, and white indicates no significant change.

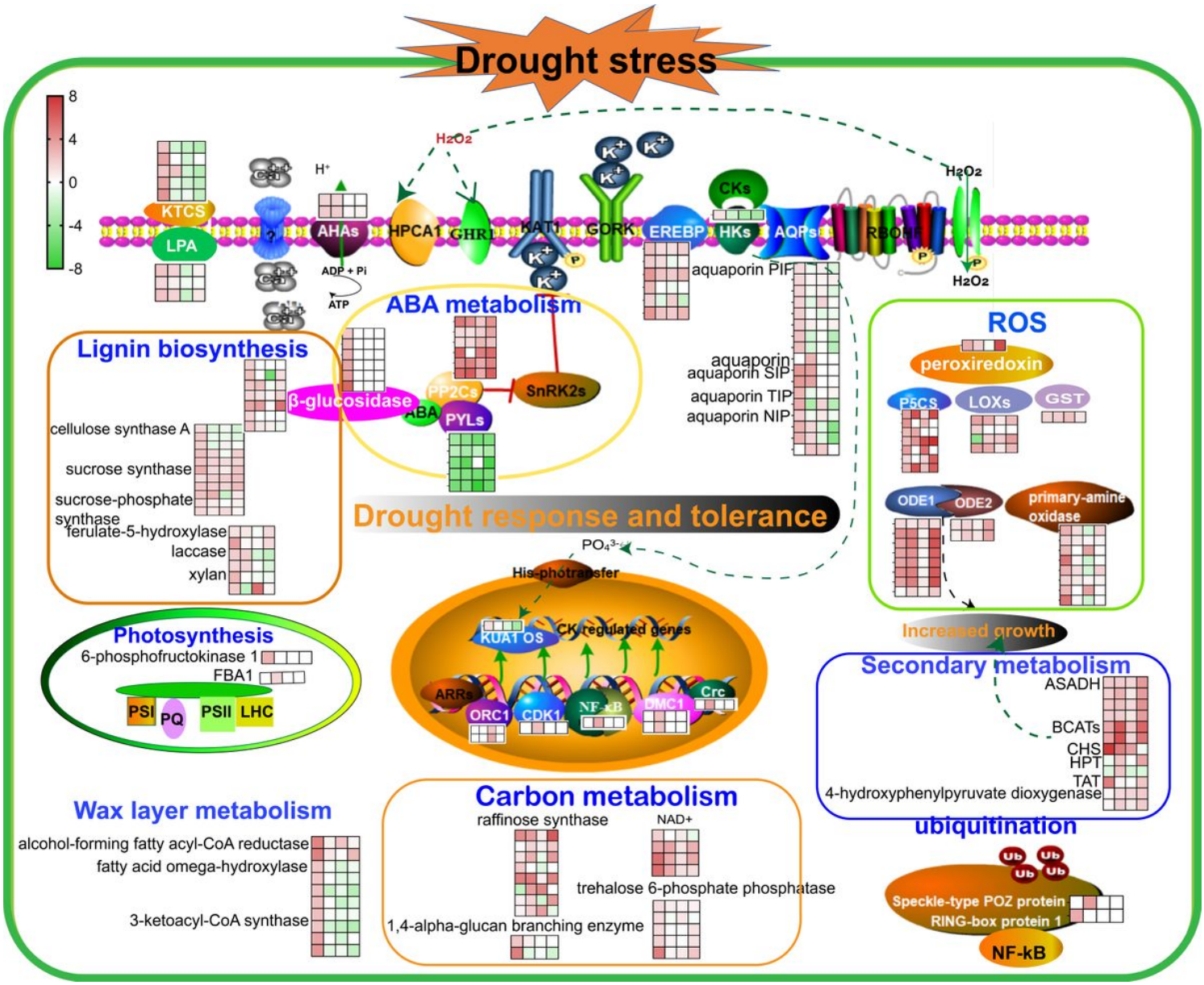

Figure 5

Hypothetical model showing the response and tolerance mechanisms of alfalfa under drought stress. The horizontal axis of the heat map represents DTR, DTL, DSR and DSL from left to right. The fold variation in DEG expression is indicated by the intensity of the box fill (see color key).

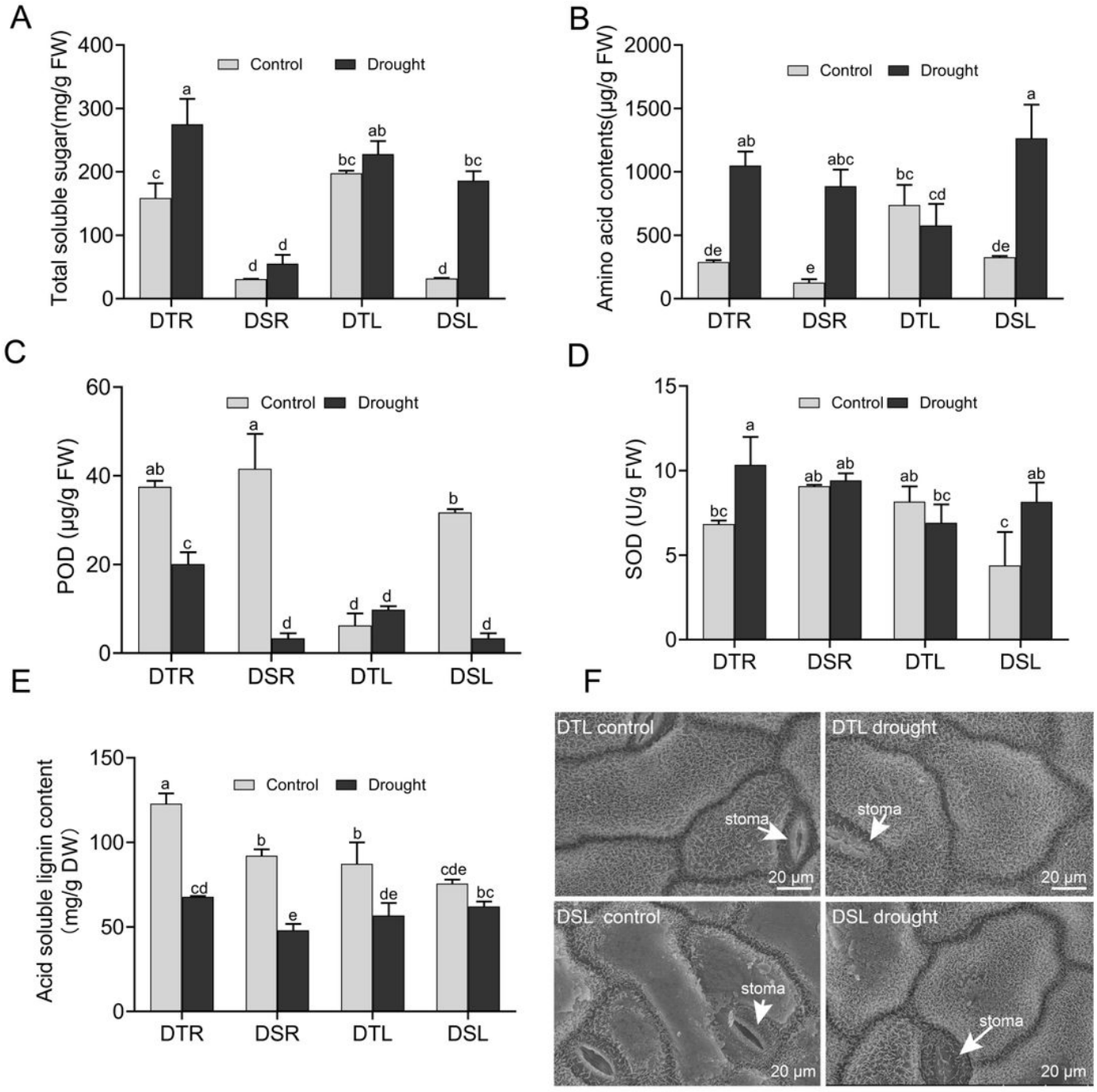

Figure 6

Effect of drought stress on physiological parameters in DT and DR genotypes. (A) Total soluble sugar contents in leaves and roots of two genotypes under control and drought conditions. (B) Amino acid contents in leaves and roots of two genotypes under control and drought conditions. (C) POD contents in leaves and roots of two genotypes under control and drought conditions. (D) SOD contents in leaves and roots of two genotypes under control and drought conditions. (E) Acid-soluble lignin content in leaves and

roots of two genotypes under control and drought conditions. (A–E) Data are mean ± SEM of three biological replicates. Lowercase letters indicate significant differences between DT and DS under the same condition as determined by one-way ANOVA followed by Dunnett's multiple comparison test. (F) Wax distribution and stomatal status of the leaf abaxial epidermis observed by electron microscopy. Photos were taken at 2000× magnification with bright field microscopy.

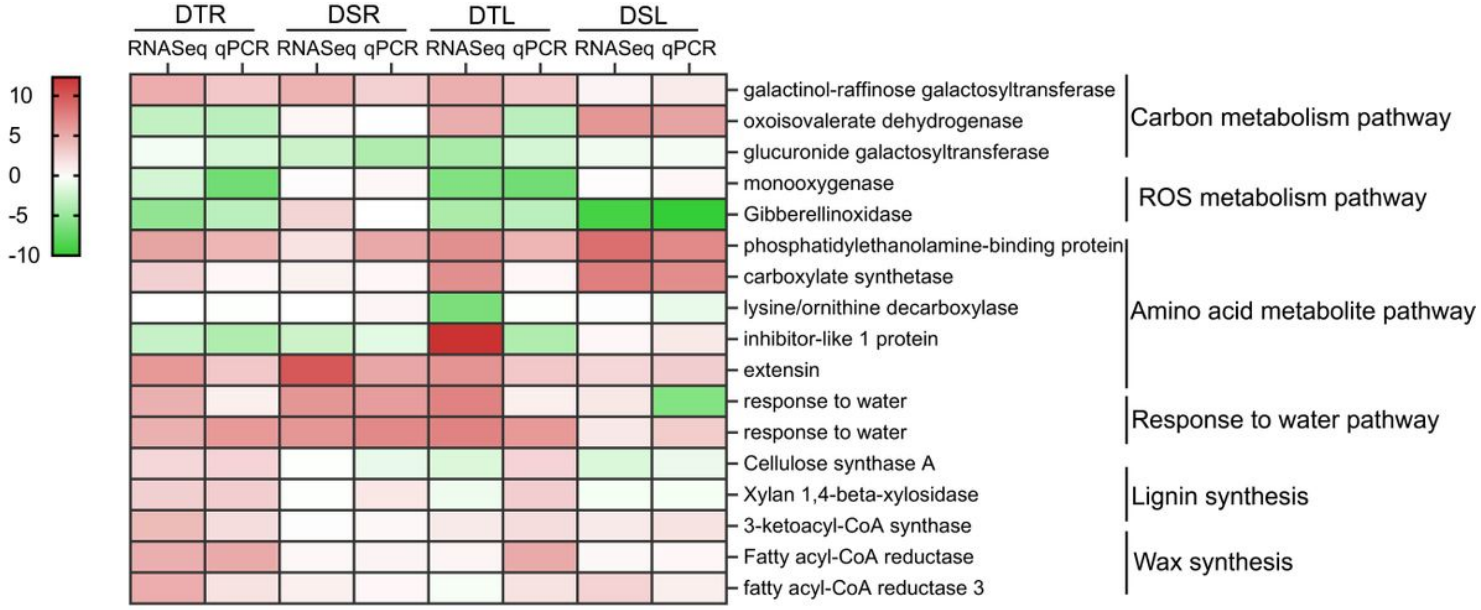

Figure 7

Correlation coefficient analysis comparing RNA-seq and qPCR fold-change data for selected DEGs. The colour scale indicates log2-transformed gene expression levels. Red indicates high transcript abundance, green indicates low transcript abundance, and white indicates no significant change.

Supplementary Files

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