Original Paper Open Access

Drought responsive genes and their functional terms identified by GS FLX Pyro sequencing in maize

Utlwang Batlang[1,2]*, Madana MR Ambavaram[1,3], Arjun Krishnan[1,4], Andy Pereira[1,5]

[1]Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, VA 24061, USA
[2]Department of Crop Science, Botswana College of Agriculture, Gaborone, Botswana
[3]Metabolix, Inc, 21 Erie Street, Cambridge, Massachusetts, MA 02139, USA
[4]Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA
[5]Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, Arkansas 72701, USA
*Corresponding author: E-mail: ubatlang@bca.bw

Abstract

Drought stress is a major challenge for the production of maize (*Zea mays* L), leading to reduced growth of aerial parts and, to a large extent, reproductive stages of development. We applied the 454 GS FLX titanium platform to identify drought differentially regulated genes in the maize vegetative and reproductive tissues. A total of 2,199 genes of which 1,284 in reproductive and 915 in vegetative tissues were identified by the platform. Quantitative RT-PCR of differentially expressed genes was carried out to confirm their expression. The results showed that the transcripts were correctly assembled and represented actively expressed genes, which genes were further subjected to gene ontology analysis for biological processes, molecular function and cell component functional terms. Significantly enriched terms indicates that catabolism of proteins and maintenance of cellular homeostasis processes were significantly enriched in the vegetative tissues, while on the other hand carbohydrate metabolism was enriched in the reproductive tissues. Photosynthesis, and energy metabolism as well as protein biosynthesis were highly repressed in both tissues. These add to the concept that drought stress target photosynthesis and causes a transition of metabolism from protein synthesis by repressing amino acid biosynthesis and translation to degradation by inducing the ubiquitin-proteasome pathway. Identified genes are potential candidates for maize improvement through transgenic and mutagenic approaches.

Keywords: maize, reproductive stage, drought stress, GS-FLX pyro-sequencing

Introduction

Drought, also known as water deficit, can result from insufficient moisture for a plant to grow adequately and complete its life cycle. Insufficient moisture can be the consequence of a shortage in rainfall, coarse textured soils that retain little water in the root zone, or drying winds (Swindale and Bidinger, 1981). Drought stress is one of the factors that most strongly limit the natural distribution of plant species, their growth and productivity worldwide (Tuberosa and Salvi, 2006; Des Marais et al, 2012). As the world population continues to grow and water resources for crop production decline, the demand for water in non-agricultural sectors is increasing (Vermeulen et al, 2013). This implies that there will be less opportunity to increase crop productivity through more irrigation, especially for dry-land crop production activities.

Reproductive development in plants show sensitivity to drought during floral initiation and the premeiotic differentiation of floral parts, with the most dramatic effects on yield recorded when stress coincides with the period between the onset of meiosis and early grain initiation (Saini, 1997). The effects of drought stress on reproductive processes in cereals has been extensively reviewed by Barnabas et al (2008). The success of cereal reproduction as well as the realization of the yield potential of a given cultivar, however, are dependent not only on the stress sensitivity of the reproductive and grain-filling developmental stages, but on overall plant growth, development and function. Efficient photosynthesis and stem reserve accumulation during the vegetative phase has a decisive role on the formation of generative organs and thus may directly affect final yield (Blum et al, 1994). Major symptoms exhibited by drought stressed plants include; photo-oxidative cellular damage, increased leaf senescence as well as reduced leaf expansion, carbon fixation, and negative effect on reproductive development. In total these processes result in reduction in carbon assimilation, which brings about the ultimate detrimental effect of reduced yield, and have been reported in cereals such as wheat (*Triticum aestivum*) (Kirigwi et al, 2004), maize (*Zea mays*) (Rebaut et al, 1997), grain sorghum (*Sorghum bicolor*) (Agboma et al, 1997), rice (Brevedan and Egli, 2003), barley (Ahmed et al, 2013), and other crop species. The emerging molecular approaches and understanding of the effect of responses and adaptation to drought is beginning

 RECEIVED 08/01/2014

to be revealed in the plant genome. Understanding of how the involved processes are affected is of particular interest for improving drought tolerance in model species and application to the wider plant kingdom.

Plants, as sessile organisms, evolved appropriate mechanisms to cope with temporary water limitations in order to ensure their survival and reproduction. Different mechanisms of drought tolerance have been reported for maize (Ribaut et al, 2009) and several plant species (Twyman et al, 2002; Nayyar, 2003). These mechanisms are often reflected at the transcription level, where the levels of mRNA related to key processes are differentially expressed (Le et al, 2012; Ranjan et al, 2012). The early events of plant responses to drought stress are the stress signal perception and subsequent transduction which lead to the activation of various molecular, biochemical and physiological responses (Hadiarto and Tran 2011). However, some of these events are post-translational, which later lead to transcriptional and translational changes. According to Yamaguchi-Shinozaki (2006), many of the genes that are known to respond to drought stress have been identified, and the products of these genes can be classified into two groups. The first group includes proteins that probably directly protect against stress such as enzymes for osmolyte biosynthesis, LEA proteins, and detoxification enzymes. The second group consists of enzymes involved in biosynthesis of signaling molecules, transcription factors, protein kinases and phosphatases. Coordinated action of these groups of genes enable plants to respond in ways that render drought tolerant or susceptible.

Genomic technologies including transcriptome analyses have provided high throughput integrated approaches to investigate global gene expression responses to drought. These transcriptome analyses has been shown to be powerful tools for the discovery of more stress-inducible genes and markers involved in stress response and tolerance, and been reported in different plant species such as Arabidopsis (Harb et al, 2010; Des Marais et al, 2012), maize (Kakumanu et al, 2012), rice (Yang et al, 2013), wheat (Ranjan et al, 2012), and soybean (Zhang et al, 2013). A cDNA microarray containing about 2,500 cDNAs from maize was used to monitor gene expression in developing maize endosperm and placenta-pedicel tissues during water deficit and re-watering (Yu and Setter, 2003). Recently, the maize expression profiles during responses to drought and other stresses were analyzed using different techniques such as oligonucleotide arrays (Hayano-Kanashiro et al, 2009), RNA-Seq (Kakumanu et al, 2012), and a customized Affymetrix microarray (Humbert al, 2013).

While these expression studies in maize in response to water stress have investigated different organs such as roots, developing kernels or particular developmental stages as revealed by the above investigations, it is imperative to use knowledge gained from this previous studies and availability of whole genome sequences and public data bases in a broader perspective. This underscores the critical role model species are playing in unraveling drought responses and how these new insights on the mechanisms of tolerance to drought are suggesting novel approaches to engineer the next generation of biotech crops.

To understand the early drought responses, we focused on the identification of genes expressed at drought sensitive maize ovaries 1-3 days after pollination (DAP) (Andersen et al, 2002) and the basal leaf meristem regulating drought-responsive vegetative leaf growth (Tardieu and Granier, 2000). To understand the functional significance of differentially regulated genes, identified genes were subjected to gene ontology analysis for biological processes (BP), molecular function (MF) and cellular components (CC). These add to the concept that drought stress target photosynthesis and causes a transition of metabolism from protein synthesis by repressing amino acid biosynthesis and translation to degradation by inducing the ubiquitin-proteasome pathway.

Materials and Methods

Maize plant material and drought stress conditions

Maize plants from inbred line B73 were grown in 10 l pots in a 1:1:1 mix of peat:vermiculite:perlite with 6 g pulverized limestone, 35 g of $CaSO_4$, 42 g of powdered $FeSO_4$, 1 g of trace fritted element (Setter et al, 2001). The plants were hand irrigated enough on daily basis to leach excess nutrient salts. Plants were supplied on weekly basis with a general purpose fertilizer, 15-16-17, (Scott-Sierra Horticultural Product Co, Marysville, OH) dissolved in water to provide 50 kg $N\text{-}P_2O_5\text{-}K_2O$ ha^{-1}. Plants were grown under well watered conditions until they reached the reproductive stage (at the onset of silk emergence), when irrigation was withheld for half of the plants. One to two days after irrigation was withheld the plants were hand pollinated and 24 hours after pollination measurements and samples were collected for RNA analysis. At this stage drought stressed plants had undergone to two or three days of drought stress and controls were well watered throughout this period.

At the end of the drought period, soil moisture content (SMC%) and the physiological measurements of chlorophyll fluorescence parameters (Fv Fm^{-1}) were determined. The basal leaf meristerm and ovary tissues corresponding with one day after pollination (Andersen et al, 2002) were sampled on half of the plants (well watered and drought stressed). In each drought stress level, samples were identified as well watered control leaf (LC), well watered control ovaries (OC), drought stressed leaf (LD) and drought stressed ovaries (OD).

GS-FLX data analysis

GS-FLX Read sequences from the two develop-

Table 1 - Effect of Drought Stress on Soil Moisture Content and Plant Physiological Responses.

Plant Species	Treatment	Soil Moisture Content (SMC) ($cm^3\ cm^{-3}$)	Relative Water Content (RWC) (%)	Chlorophyll Fluorescence ($Fv\ Fm^{-1}$)
Maize	Control	0.46a	96.7a	0.76a
	Drought stressed	0.12b	77.7b	0.64b
	LSD (0.05)	0.012	9.88	0.001
	P value	<0.0001	<0.0001	<0.0001

mental stages of four libraries (LC, LD, OC, OD), that were trimmed for low quality and primer sequences were masked for plant repeat sequences. Reads were unmasked (non-repeat) sequence length of at least 100 bp were used for further analysis. These reads were mapped to the maize inbred B73 genomic cDNA sequences (www.maizesequence.org) by BLASTN. Reads mapping with BLASTN e-values $< 10^{-5}$ and bitscore > 100 were used for further analyses. The number of reads mapping to the total length of the maize gene, and separately to the last 1,000 bp of the gene sequence were recorded. In a given library genes with three or more matching reads were considered present in that library. Comparisons were made between the libraries; MLC against MLD and MOC against MOD, and genes present in at least one of them were used calculate the log2 ratio based on the number of hits within the last 1,000 bp of a gene.

Quantitative realtime-PCR (qRT-PCR) analysis

Differentially expressed genes were validated by performing qRT-PCR on a set of selected transcripts. This set was chosen to represent the genes which are specific to different developmental tissue (Vegetative and Reproductive). The PCR primers were designed using Molecular beacon software, primer length 20 - 25 nucleotides, and an expected amplicon size of 100 - 125bp. The comparative Ct method of quantitation was used with the *Tubulin* gene as a reference. The relative fold-change for each of the selected genes was detected from both the drought and control plants. Three independent biological replicates of each sample and two technical replicates of each biological replicate were used for real-time PCR analysis. For each sample, 1 µg total RNA from one of the biological replicates was converted into cDNA using oligodT 15-mer (Promega) and Superscript III reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). This cDNA was diluted to 250 µl in sterile water. Validation experiments were performed on 5 to 6 log dilutions of each of the target genes together with the Actin reference to determine if the amplification efficiencies were equal. Triplicate qRT-PCR reactions were performed using iQ™SYBR® Green Supermix (Bio-Rad, Hercules, CA), The PCRs were performed in a Bio-Rad iQ5™ thermocycler (Bio-Rad, Hercules, CA). The temperature regime used was 95°C for 10 m followed by 40 cycles of 30 s at 95°C, 45 s at 55°C and 45 s at 72°C. Melting curve analysis by applying increasing temperature from 55°C to 95°C (0.5°C $10\ s^{-1}$) and gel and gel electrophoresis of final product confirmed single amplicons. Negative control reactions using untranscribed RNA were also run to confirm absence of genomic DNA. The relative fold change for a particular target was determined by comparing the Ct values for the treatment with that of the control. The Ct values were normalized using the Ct reference (tubulin) prior to comparison.

Gene ontology analysis

To understand the functional significance of differentially regulated genes Fisher's exact test was performed for declaring a GO (Gene Ontology) category as significantly over-represented (Benjamini-Yekutieli method for controlling FDR, adjusted p-value < 0.05) using PlantGSEA toolkit (Yi et al, 2013). Terms with P < 0.05 were declared significantly enriched.

Results and Discussion

Physiological responses to drought stress and identification of differentially regulated genes

The imposing of drought stress significantly reduced soil moisture content from 0.46 $cm^3\ cm^{-3}$ (controls) to 0.12 $cm^3\ cm^{-3}$ for drought stressed treatment (Table 1), a response of 78.3% reduction. The soil water potential of each treatment was 0.003 Mpa (controls) and 1.02 for drought stressed treatment. In response to this decline in soil moisture content, leaf relative water content dropped from 96.7% (controls) to 77.7% (drought stressed), representing a 20% reduction in the ratio. Chlorophyll fluorescence significantly dropped by 13% from 0.76 to 0.64. The level of drought stress at the soil water potential can be described as severe as previously determined by Chen et al (2012). Previous and similar studies revealed that this level of drought stress inhibited photosynthesis, disrupted carbohydrate metabolism and decreased kernel number (Zinselmeier et al, 1994, 1995; Chen et al, 2012).

Global gene expression analysis in maize from the two tissues (leaf meristem and reproductive) using GS-FLX pyrosequencing enables a genome wide comparison of differentially expressed genes under drought stress conditions. Further, the system identified 2,199 genes in the LC, LD, OC, and OD libraries (Table 2). Out of these, 1,284 genes were from the leaf libraries and 915 responded to drought. The accuracy of the GS-FLX pyrosequencing was verified by selecting ten genes that were previously associated with dehydration stress (drought, cold and salin-

Table 2 - Rice - maize gene orthologs identified by the GS-FLX pyrosequencing platform.

Library	Read Number[$]	Maize Gene Best Hits[£]	Genes Present[‡]	Comparable Genes[¥]
LC	14,472	3,424	569	
LD	17,718	4,420	863	1,284
OC	39,768	390	138	
OD	16,499	4,244	837	915

[$]Number of reads with at least 100 bp were used for analysis.
[£]Reads that mapped to maize (B73) genomic cDNA sequences with a BLASTn e-value < 10^{-5} and mapping to the total length of maize gene, and separately to 1,000 bp of the gene sequence.
[‡]Genes with at least three read matches are considered present.
[¥]Genes present in at least one of the libraries are compared for their expression level.

ity stress) and subjecting them to qRT-PCR (Table 3). These genes were found to be drought responsive in maize (Benesova et al, 2012; Peng et al, 2012) its relatives (Aprile et al, 2013) and other plant species (Kim et al, 2004; Bhaskara et al, 2012). The observed Pearson correlation between GS-FLX $\log_2$ ratio and qRT-PCR data was 0.701 (Ddata not shown). In view of these identified genes were subjected to gene ontology analysis.

Up-regulated genes in the vegetative tissues indicate the effect of drought on protein degradation and re-establishment of cellular homeostasis.

Gene ontology (GO) analysis for biological processes of the up-regulated genes revealed that catabolism of proteins (GO:0030163) was significantly enriched in the drought-stressed vegetative tissues (Table 4). The gene, 26S-protease regulatory subunit (GRMZM2G137528), is part of the ubiquitin- and proteasome proteolytic pathway involved in the turnover of misfolded proteins and hormone-mediated signal transduction (Ingvardsen and Veierskov, 2001; Lyzenga and Stone, 2011). Protein degradation is a normal cellular activity, but an increase is degradation in response to drought can be interpreted as the result of excessive protein damage. This turnover is necessary for the removal of abnormal or damaged proteins and for altering the balance of proteins and, in a worst-case scenario, apoptotic degradation of damaged cells. On the other hand protein degradation can be an adaptive response to drought. This was demonstrated by drought tolerance observed in Arabidopsis (Lee et al, 2009) and *Nicotiana tabacum* (Guo et al, 2009) as a result of enhancement of the protein degradation processes involved.

The processes, cell redox homeostasis (GO:0045454) and regulation of biological quality (GO:0065008) are generally involved in the maintenance of cellular homeostasis. These processes are deemed important as stress adaptive responses and signalling for the reestablishment of cellular homeostasis under stress conditions, to control and repair stress damages, and coordinate cell division and expansion to levels suitable for water deficit. Therefore, once cellular homeostasis is reestablished, stress injury would be reduced. Genes involved in these processes belong to the theoredoxin superfamily. These genes are thioredoxin_H type (GRMZM2G082886), theoredoxin_M type (GRMZM2G170008), glutaredoxin subgroup I (GRMZM2G150295) (Table 4). Drought stress can result in changes in the chloroplast, mitochondria, and cytosol redox state, leading to oxidative damage to biological membranes and proteins. Several lines of evidence indicate that theoredoxins can relieve oxidative stress by modulating both the activity of enzymes scavenging ROS and other functions related to control of cell redox homeostasis (Broin and Rey, 2003; Dos Santos and Rey, 2006) by re-reducing the oxidized -S-S- groups in enzymes involved in metabolism (Buchanan and Balmer, 2005). Induction of these genes by drought likely results from the changes in cellular redox state, and the proteins participate in response to oxidative stress within organelles (chloroplasts and mitochondria) and in the cytosol upon oxidative stress. It is

Table 3 - Maize-rice ovary differentially expressed orthologous genes in both the leaf and ovary libraries.

Maize-ID	Annotation	Log_2 Ratio[$]	Relative Expression[&]
GRMZM2G136364	Lipid binding protein	7.9432	0.77 (0.31)
GRMZM2G147014	Dehydrin_COR14	3.6825	0.77 (0.30)
GRMZM2G134628	ABI-2 (PP2C)	0.5473	0.53 (0.79)
GRMZM2G173124	Zinc finger protein_CCCH type	2.6236	1.26 (0.30)
GRMZM2G129018	CIPK-like protein_1	1.0623	1.02 (0.78)
GRMZM2G137839	Class_1 heat shock protein	1.5079	0.90 (0.25)
GRMZM2G046382	Cytosolic ascorbate peroxidase	1.1322	1.04 (0.25)
GRMZM2G129246	Hydroxyacid oxidase_1	-1.1399	-1.00 (0.19)
GRMZM2G139467	Cytochrome_P450_93A3	-0.4515	-0.89 (0.26)
GRMZM2G162200	RUBISCO activase	-1.6590	-2.34 (0.31)

[$]The log2 ratio as determined by rice microarray.
[&]Relative expression by qRT-PCR analysis of the maize reproductive tissue.

Table 4 - Enriched gene ontology terms in vegetative tissues in response to drought stress.

Gene ID	GO Term Description and Gene annotation	Category	P Value	FDR
	up-regulated genes and functional categories			
GO:0045454	cell redox homeostasis	GO_BP	4.14E-06	6.08E-05
GRMZM2G170008	thioredoxin_M type, chloroplast precursor			
GRMZM2G150295	Grx_C2.2-glutaredoxin subgroup I			
GRMZM2G082886	theoredoxin_H type			
GO:0030163	protein catabolic process	GO_BP	4.23E-03	0.0207
GRMZM2G137528	26S protease regulatory subunit 8			
GO:0006662	glycerol ether metabolic process	GO_BP	1.36E-03	0.01
GRMZM2G170008	thioredoxin_M type			
GO:0019288	Isopentenyldiphosphate biosynthetic process mevalonate-independent pathway	GO_BP	1.77E-4	1.92e-3
GRMZM2G027059	4-hydroxy-3-mthylbut-2-enyl disphosphatereductase			
	down-regulated genes and functional categories			
GO:0015979	photosynthesis	GO_BP	4.82E-16	6.02E-14
GRMZM2G024150	photosystem I reaction centre subunit			
GRMZM2G139803	ferridoxin-theoredoxinreductase, variable chain			
GRMZM2G016677	oxygen evolving enhancer protein 2			
GRMZM2G016066	photosystem I reaction center subunit IVA			
GRMZM2G132506	photosystem II reaction centre W protein			
GRMZM2G085646	photosystem I reaction centre subunit III			
GRMZM2G026015	photosystem I reaction centre subunit XI			
GRMZM2G080107	photosystem I reaction centre subunit N			
GRMZM2G021256	oxygen evolving enhancer 3 protein			
GO:0006412	translation	GO_BP	9.85E-06	6.15E-04
GRMZM2G153476	30S ribosomal protein S13			
GRMZM2G113873	50S ribosomal protein L6			
GRMZM2G176820	50S ribosomal protein L11, chloroplast precursor			
GRMZM2G018403	50S ribosomal protein L21			
GRMZM2G170870	ribosomal protein L6			
GRMZM2G042061	50S ribosomal protein L28, chloroplast precursor			
GRMZM2G113414	translation initiation factor SUI1 homolog2			
GO:0006414	translational elongation	GO_BP	7.63E-04	0.0268
GRMZM2G179976	60S acidic ribosomal protein Po			
GRMZM2G106061	elongation factor Tu			
GO:0019288	isopentenyldiphosphate biosynthetic process	GO_BP	8.57E-04	0.0268
GRMZM2G027059	4-hydroxy-3-mthylbut-2-enyl disphosphatereductase			
GO:0003735	structural constituent of ribosome	GO_MF	2.75E-05	2.43E-3
GRMZM2G109165	50S Ribosomal protein L3, chloroplast precursor			
GRMZM2G153476	30S ribosomal protein S13			
GRMZM2G176820	50S ribosomal protein L11, chloroplast precursor			
GRMZM2G018403	50S ribosomal protein L21			
GRMZM2G179976	60S acidic ribosomal protein Po			
GRMZM2G170870	ribosomal protein L6			
GRMZM2G042061	elongation factor Tu			
GO:0030170	pyridoxal phosphate binding	GO_MF	7.23E-06	1.28E-03
GRMZM2G082185	Cysteine synthase, mitochondrial precursor			
GRMZM2G108514	Tyrosine decarboxylase 4			
GRMZM2G078143	Serine hydroxylmethyltransferase, mitochondrial precursor			
GRMZM2G113873	Cystathionine beta-lyase			
GO:0009538	photosystem I reaction center	GO_CC	8.04E-11	3.33E-09
GRMZM2G024150	photosystem I reaction centre subunit			
GRMZM2G016066	photosystem I reaction center subunit IVA			
GRMZM2G085646	photosystem I reaction centre subunit III			
GRMZM2G026015	photosystem I reaction centre subunit XI			
GO:0009654	oxygen evolving complex	GO_CC	7.74E-07	1.60E-05
GRMZM2G016677	Oxygen evolving enhancer protein 2			
GRMZM2G021256	oxygen evolving enhancer 3 protein			
GRMZM2G132506	photosystem II reaction centre W protein			
GO:0005622	intracellular	GO_CC	1.80E-03	0.0124
GRMZM2G109165	50S Ribosomal protein L3, chloroplast precursor			
GRMZM2G153476	30S ribosomal protein S13			
GRMZM2G018403	50S ribosomal protein L21			
GRMZM2G179976	60S acidic ribosomal protein Po			
GRMZM2G170870	ribosomal protein L6			
GRMZM2G106061	Elongation factor Tu			
GRMZM2G042061	50S Ribosomal protein L28, chloroplast precursor			
GO:0005840	ribosome	GO_CC	2.11E-05	2.90E-04
GRMZM2G109165	50S Ribosomal protein L3, chloroplast precursor			
GRMZM2G153476	30S ribosomal protein S13			
GRMZM2G176820	50S ribosomal protein L11, chloroplast precursor			
GRMZM2G018403	50S ribosomal protein L21			
GRMZM2G179976	60S acidic ribosomal protein Po			
GRMZM2G170870	ribosomal protein L6			
GRMZM2G042061	elongation factor Tu			

Table 4 - continued.

Gene ID	GO Term Description and Gene annotation	Category	P Value	FDR
GO:0019898	extrinsic to membrane	GO_CC	1.02E-04	1.05E-03
GRMZM2G016677	Oxygen evolving enhancer protein 2			
GRMZM2G021256	oxygen evolving enhancer 3 protein			
GO:0009536	plastid	GO_CC	2.62E-04	2.16E-03
GRMZM2G139803	ferridoxin-theoredoxinreductase, variable chain			
GRMZM2G016066	photosystem I reaction center subunit IVA			
GO:0009288	GO:0009288 bacterial-type flagellum			
GRMZM2G085747	NAD-dependent malic enzyme 59 Kda, mitochondria precursor			

The GO category, P value, and false discovery rate (FDR) for each significantly enriched GO term are shown.

also worth noting the up-regulation of the Isopentenyl diphosphate biosynthetic process (GO:0019288). Isopentenyl disphosphate (IPP) is an intermediate in the biosynthesis of ABA in plants (Milborrow, 2000), which confirms the well-established role of ABA in plant responses to drought stress.

Up-regulated genes in the reproductive tissues effecting carbohydrate metabolism

In the reproductive tissue, enriched biological processes were; carbohydrate catabolism (GO:0030163) and cellulose biosynthesis (GO:0030244). Genes involved in these process are periplasmic beta-glucosidase precursor (GRMZM2G147687), glucan endo-1,3-beta-glucosidase 7 precursor (GRMZM2G127117), beta1,3;1,4 glucan synthase (GRMZM2G122277) (Table 5). Beta-glucosidases are involved in the selective cleavage of glucose from polysaccharides; and the glucose may then re-enter sugar-nucleotide interconversion pathways (Leah et al, 1995), contributing to the synthesis of new polysaccharides and other types of polymers. Most ß-glucosidases also possess glucotransferase activity, which may enable them to act on glucose units to form a diversity of oligosaccharides (Leah et al, 1995; Amiard et al, 2003), and other carbohydrates that may be important for drought tolerance. Previous studies in maize show that drought stress inhibits invertases and that glucose from sucrose is rendered unavailable under such conditions (Zinselmeier et al, 1995). This could represent an attempt by plant cells to derive glucose from other sources for primary metabolism to continue during stress. Perhaps the cleaved glucose may be used in the synthesis of 1,3:1,4-glucan (callose), which could be an indication that the chemical composition of the cell wall is altered in these tissues in response to drought as reported by Ingram and Bartels (1996). The results suggest that carbohydrate metabolism may be one of the targets of drought stress or an adaptive response in reproductive tissues.

Down-regulated Genes in the Vegetative tissue Indicates the Sensitivity of Photosynthesis and Proteosynthesis to Drought Stress.

Analysis of down-regulated as well as up-regulated genes in drought stress is important for understanding general biological responses to stress. Identified genes were further analyzed to identify the biological processes affected significantly under drought. Photosynthesis (GO:0015979 and GO:0019684) processes and energy metabolism (GO:0006091) were among the down-regulated processes (Table 5). The same processes are also down-regulated in the reproductive tissues. Genes taking part in these processes are; photosystem I reaction center subunits (GRMZM2G024150), photosystem II and I reaction center subunits, and photosystem II oxygen-evolving enhancer proteins. Generation of precursor metabolites and energy (GO:0042592) in which two ATP synthase genes take part was also repressed by drought stress. These genes constitute part of the processes involved in the light reactions of photosynthesis from oxygen evolution to ATP synthesis by the ATP synthase. These results echo the reduced photosynthesis indirectly determined by chlorophyll fluorescence (Table 1), which is consistent with many previous studies showing that drought stress inhibits photosynthesis. Down-regulation of photosynthetic genes under drought stress has been observed in rice (Hazen et al, 2005), barley (Ozturk et al, 2002; Talame et al, 2007), and soybean (Ranjan et al, 2012). The down-regulation of photosynthesis can arise due to oxidative stress, which cause damage to the photosynthetic machinery. The down-regulation of the light reactions can be an adaptive mechanism to reduce further damage to the machinery by excessive light under drought stress.

Processes involved in amino acid biosynthesis, such as cellular amino acid and derivative metabolic process (GO:0006519) and organic acid metabolism (GO:0006082) and translation (GO:0006412), were also down-regulated as a result of drought stress in vegetative tissue. This shows that important processes of protein biosynthesis, which are biosynthesis of amino acids and translation of gene transcripts into proteins, are repressed. For example genes involved in translation, translation initiation factor SU1 (GRMZM2G113414) and translation elongation factor (GRMZM2G106061) (Table 4) or their homologs have been reported to be repressed by drought in (Sigh et al, 2004). It is therefore speculated that the decline in protein biosynthesis (proteosynthesis) could be caused by protein degradation and the limited availability of amino acids associated with drought-induced inhibition of photosynthesis.

Table 5 - Differentially regulated genes in the maize ovaries.

Gene ID	GO Term Description and Gene annotation	Category	P Value	FDR
	up-regulated genes and functional categories			
GO:0005975	Carbohydrate metabolic process	GO_BP	4.14E-06	6.08E-05
GRMZM2G127117	glucan endo-1,3-beta-glucosidase 7 precursor			
GRMZM2G147687	periplasmic beta-glucosidase precursor			
GO:0030244	cellulose biosynthetic process	GO_BP	5.03E-03	7.54E-03
GRMZM2G122277	beta1,3;1,4 glucan synthase			
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	GO_MF	6.72e-4	2.02e-3
GRMZM2G127117	glucan endo-1,3-beta-glucosidase 7 precursor			
GRMZM2G147687	periplasmic beta-glucosidase precursor			
	down-regulated genes and functional categories			
GO:0015979	photosynthesis	GO_BP	0.0114	0.0114
GRMZM2G012397	Photosystem I reaction center subunit psaK, chloroplast precursor			
GO:0009522	photosystem I	GO_CC	1.51E-03	4.54E-03
GRMZM2G012397	Photosystem I reaction center subunit psaK, chloroplast precursor			

The GO category, P value, and false discovery rate (FDR) for each significantly enriched GO term are shown.

Conclusions

This study adds to the concept that drought stress causes a transition of metabolism from protein synthesis by repressing amino acid biosynthesis and translation to degradation by inducing the ubiquitin-proteasome pathway. In addition, photosynthesis is severely inhibited by repression of genes involved in both the light and dark reactions. Identified genes will not only facilitate understanding of genetic basis of drought stress response, but also accelerate genetic improvement transformation and mutagenesis, marker-assisted selection in maize.

References

Agboma PC, Jones MGK, Peltonen-Sainio P, Rita H, Pehu E, 1997. Exogenous glycinebetaine enhances grain yield of maize, sorghum and wheat grown under two supplementary watering regimes. J Agron Crop Sci 178: 29-37

Ahmed IM, Cao F, Zhang M, Chen X, Zhang G, Wu F, 2013. Difference in yield and physiological features in response to drought and salinity combined stress during anthesis in Tibetan wild and cultivated barleys. PLoS ONE 8: e77869

Amiard V, Morvan-Bertrand A, Billard J-P, Huault C, Keller F, Prud'homme M-P, 2003. Fructans, but not the sucrosyl-galactosides, raffinose and loliose, are affected by drought stress in perennial ryegrass. Plant Physiol. 132: 2218-2229

Anderson MN, Asch F, Wu Y, Jesnsen CR, Naested H, Mogensen VO, Koch KE, 2002. Soluble invertase expression is an early target of drought stress during the critical, abortion-sensitive phase of young ovary development in maize. Plant Physiol 130: 591-604

Aprile A, Havlickova L, Panna R, Marè C, Borrelli GM, Marone D, Perrotta C, Rampino P, De Bellis L, Curn V, Mastrangelo AM, Rizza F, Cattivelli L, 2013. Different stress responsive strategies to drought and heat in two durum wheat cultivars with contrasting water use efficiency. BMC Genomics 14: 821

Barnabas B, Jager K, Feher A, 2008. The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ 31: 11-38

Benesova M, Hola D, Fischer L, Jedelsky PL, Hnilicka F, Wilhelmova N, Rothova O, Kocova M, Prochazkova D, Honnerova J, Lenka F, Hnilickova H, 2012. The Physiology and Proteomics of Drought Tolerance in Maize: Early Stomatal Closure as a Cause of Lower Tolerance to Short-Term Dehydration? PLoS ONE 7: e38017

Bhaskara GB, Nguyen TT, Verslues PE, 2012. Unique drought resistance functions of the highly ABA-induced clade A protein phosphatase 2Cs. Plant Physiology 160: 379-395

Blum A, 1998. Improving wheat grain filling under stress by stem reserve mobilisation. Euphytica 100: 77-83

Brevedan RE, Egli DB, 2003. Short periods of water stress during seed filling, leaf senescence, and yield of soybean. Crop Sci 43: 2083-2088

Broin M, Rey P, 2003. Potato plants lacking the CDSP32 plastidic thioredoxin exhibit overoxidation of the BAS1 2-cysteine peroxiredoxin and increased lipid peroxidation in thylakoids under photooxidative stress. Plant Physiol 132: 1335-1343

Buchanan B, Balmer Y, 2005. Redox regulation: a broadening horizon. Ann Rev Plant Biol 56: 187-220

Chen J, Xu W, Velten Z, Xin Z, Stout J, 2012. Characterization of maize inbred lines for drought and heat tolerance. J Soil Water Conserv 67: 354-364

Des Marais DL, McKay JK, Richards JH, Sen S, Wayne T, Juenger TE, 2012. Physiological genomics of response to soil drying in diverse Arabidopsis accessions. Plant Cell 24: 893-914

Dos Santos CV, Rey P, 2006. Plant thioredoxins are key actors in the oxidative stress response. Trends Plant Sci 11: 1329-1334

Guo P, Baum M, GrandoS, Ceccarelli S, Bai G, Li R, von Korff M, Vaeshney KK, Graner A, Valkoun J, 2009. Differentially expressed genes between drought-tolerant and drought sensitive barley genotypes in response to drought stress during

reproductive stage. J Exp Bot 60: 3531-3544
Guo Q, Zhang J, Gao Q, Xing S, Li F, Wang W, 2009. Drought tolerance through overexpression of monoubiquitin in transgenic tobacco. Journal of Plant Physiology 165: 1745-1755
Hadiarto T, Tran LS, 2011. Progress studies of drought-responsive genes in rice. Plant Cell Rep 30: 297-310
Harb A, Krishnan A, Ambavaram MM, Pereira A, 2010. Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. Plant Physiol 154: 1254-1271
Hayano-Kanashiro C, Calderon-Vazquez C, Ibarra-Laclette E, Herrera-Estrella L, Simpson J, 2009. Analysis of gene expression and physiological responses in three Mexican maize landraces under drought stress and recovery irrigation. PLoS One 4: e7531
Hazen SP, Pathan MS, Sanchez A, Baxter I, Dunn M, Estes B, Chang HS, Zhu T, Kreps JA, Nguyen HT, 2005. Expression profiling of rice segregating for drought tolerance QTLs using a rice genome array. Funct Integr Genomics 5: 104-116.
Humbert S, Subedi S, Cohn J, Zeng B, Bi YM, Chen X, Zhu T, McNicholas PD, Rothstein SJ, 2013. Genome-wide expression profiling of maize in response to individual and combined water and nitrogen stresses. BMC Genomics 14: 3
Ingram J, Bartel D, 1996. The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol.47: 377-403
Invardsen C, Veirskov B (2001) Ubiquitin- and proteasome-dependent proteolysis in plants. Physiol Plant 112: 451-459
Kakumanu A, Ambavaram MM, Klumas C, Krishnan A, Batlang U, Myers E, Grene R, Pereira A, 2012. Effects of drought on gene expression in maize reproductive and leaf meristem tissue revealed by RNA-Seq. Plant Physiol 160: 846-867
Kim YJ, Kim JE, Lee J-H, Lee MH, Ho Won Jung HW,Bahk YY, Hwang BK, Hwang I, Kim WT, 2004. The Vr-PLC3 gene encodes a putative plasma membrane-localized phosphoinositide-specific phospholipase C whose expression is induced by abiotic stress in mung bean (*Vigna radiata* L). FEBS Letters 556, 127-136
Kirigwi, FM, Van Ginkel M, Trethowan R, Sears RG, Rajaram S, Paulsen GM, 2004. Evaluation of selection strategies for wheat adaptation across waterregimes. Euphytica 135, 361-371
Le DT, Nishiyama R, Watanabe Y, Tanaka M, Seki M, Ham le H, Yamaguchi-Shinozaki K, Shinozaki K, Tran LS, 2012. Differential gene expression in soybean leaf tissues at late developmental stages under drought stress revealed by genome-wide transcriptome analysis. PLoS One 7: e49522
Leah R, Kigel J, Svendsen IB, Mundy J, 1995. Biochemical and molecular characterization of barley seed b-glucosidases. J Biol Chem 270: 15789-15797
Lee HK, Cho SK, Son O, Xu Z, Hwang I, Woo Taek Kim WT, 2009. Drought stress-induced Rma1H1, a RING Membrane-anchor E3 ubiquitin ligase homolog, regulates Aquaporin levels via ubiquitination in transgenic arabidopsis plants. Plant Cell 21: 622-641
Lyzenga WJ, Stone SL, 2011. Abiotic stress tolerance mediated by protein ubiquitination. Journal of Experimental Botany. Doi:10.1093/jxb/err310
Milborrow BV, 2000. The pathway of biosynthesis of abscisic acid in vascular plants: a review of the present state of knowledge of ABA biosynthesis. Journal of Experimental Botany, 52: 1145-1164
Nayyar H, 2003. Variation in osmoregulation in differentially drought-sensitive wheat genotypes involves calcium. Biol Plant 47: 541-547
Ozturk ZN, Talame V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ, 2002. Monitoring large scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol 48: 551-573
Peng X, Zhao Y, Cao J, Zhang W, Jiang H, Li X, Ma Q, Zhu s, Cheng B, 2012. CCCH-Type Zinc Finger Family in Maize: Genome-Wide Identification, Classification and Expression Profiling under Abscisic Acid and Drought Treatments. PLoS ONE 7: e40120
Ranjan A, Nigam D, Asif MH, Singh R, Ranjan S, Mantri S, Pandey N, Trivedi I, Rai KM, Jena SN, Koul B, Tuli R, Pathre UV, Sawant SV, 2012. Genome wide expression profiling of two accession of *G. herbaceum* L in response to drought. BMC Genomics 13: 94
Ribaut J, Jiang C, Gonzalez-de-Leon D, Edmeades G, Hoisington D, 1997. Identification of quantitative trait loci under drought conditions in tropical maize. 2. Yield components and marker-assisted selection strategies. Theor Appl Genet 94: 887-896
Ribaut R-M, Betran J, Monneveux P, Setter T, 2009. Drought tolerance in maize. In: Handbook of maize. Bennetzen JL, Hake SC eds. Springer Science, NY
Saini H, 1997. Effect of water stress on male gametophyte development in plants. Sexual Plant Reprod 10: 67-73
Setter TL, Flannigan BA, Melkonian J, 2001. Loss of kernel set due to water deficit and shade in maize: carbohydrate supplies, abscisic acid, and cytokinins. Crop Sci 41:1530–1540
Singh BN, Mishra RN, Agarwal PK, Goswami M, Nair S, Sopory SK, Reddy MK, 2004. A pea chloroplast translation elongation factor that is regulated by abiotic factors. Biochemical and Biophysical Research Communications 320: 523-520
Swindale LD, Bidinger FR, 1981. The human consequences of drought and crop research priorities

for their alleviation, pp. 2-13. In: The physiology and biochemistry of drought resistance in plants. Paleg LG, Aspinal D eds. Academic Press. Sydney

Talame V, Ozturk NZ, Bohnert HJ, Tuberosa R, 2007. Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. J Exp Bot 58: 229-240

Tardieu F, Granier C, 2000. Quantitative analysis of cell division in leaves: methods, developmental patterns and effects of environmental conditions. Plant Mol Biol 43: 555-567

Tuberosa R, Salvi S, 2006. Genomics approaches to improve drought tolerance in crops. Trends Plant Sci 11:405-412

Twyman RM, Stoger E, Kohli A, Capell T, Christou P, 2002. Selectable and screenable markers for rice transformation, pp. 1-5. In: Molecular methods of plant analysis; testing for genetic manipulation in plants. Jackson JF, Liskens HF, Inman RB eds. Springer-Verlag, Berlin, Germany

Vermeulen SJ, Challinor AJ, Thornton PK, Campbell BM, Eriyagama N, Vervoort M, Kinyangi J, Jarvis A, Laderach P, Ramirez-Villegas J, Nicklin K, Hwakins E, Smith DS, 2013. Addressing uncertainty in adaptation planning for agriculture. Proc Natl Acad Sci USA 110: 8357-8362

Yamaguchi-Shinozaki K, Shinozaki K, 2006. Transcriptional regulatory network in cellular responses and tolerance to dehydration and cold stresses. Ann Rev Plant Biol 57: 781-803

Yang Y, Li Y, Wu C, 2013. Genomic resources for functional analyses of the rice genome. Curr Opin Plant Biol 16: 157-163

Yi X, Du Z, Su Z, 2013. PlantGSEA: a gene set enrichment analysis toolkit for plant community. Nucleic Acids Res. 41, W98–W103. doi: 10.1093/nar/gkt281

Yu L-X, Setter TL, 2003. Comparative transcriptional profiling of placenta and endosperm in developing maize kernels in response to water deficit. Plant Physiol 131: 568-582

Zhang DY, Ali Z, Wang CB, Xu L, Yi JX, Xu ZL, Liu XQ, He XL, Huang YH, Khan IA, Trethowan RM, Ma HX, 2013. Genome-wide sequence characterization and expression analysis of major intrinsic proteins in soybean (*Glycine max* L.) PLoS ONE 8: e56312

Zinselmeier C, Westgate ME, Jones RJ, 1994. Kernel set at low water potential does not vary with source/sink ratio in maize. Crop Sci 35: 158-163

Zinselmeier C, Westgate ME, Schussler JR, Jones RJ, 1995. Low water potential disrupts carbohydrate metabolism in maize (*Zea mays* L) ovaries. Plant Physiol 107: 385-391