Effects of Drought on Gene Expression in Maize Reproductive and Leaf Meristem Tissue Revealed by RNA-Seq[1][W][OA]

Akshay Kakumanu[2], Madana M.R. Ambavaram[2,3], Curtis Klumas, Arjun Krishnan[4], Utlwang Batlang[5], Elijah Myers, Ruth Grene*, and Andy Pereira

Virginia Bioinformatics Institute (A.Ka., M.M.R.A., A.Kr., U.B., A.P.), Department of Plant Pathology (A.Ka., R.G.), and Genetics, Bioinformatics, and Computational Biology Program (C.K., E.M.), Virginia Tech, Blacksburg, Virginia 24061; and Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, Arkansas 72701 (A.P.)

Drought stress affects cereals especially during the reproductive stage. The maize (*Zea mays*) drought transcriptome was studied using RNA-Seq analysis to compare drought-treated and well-watered fertilized ovary and basal leaf meristem tissue. More drought-responsive genes responded in the ovary compared with the leaf meristem. Gene Ontology enrichment analysis revealed a massive decrease in transcript abundance of cell division and cell cycle genes in the drought-stressed ovary only. Among Gene Ontology categories related to carbohydrate metabolism, changes in starch and Suc metabolism-related genes occurred in the ovary, consistent with a decrease in starch levels, and in Suc transporter function, with no comparable changes occurring in the leaf meristem. Abscisic acid (ABA)-related processes responded positively, but only in the ovaries. Related responses suggested the operation of low glucose sensing in drought-stressed ovaries. The data are discussed in the context of the susceptibility of maize kernel to drought stress leading to embryo abortion and the relative robustness of dividing vegetative tissue taken at the same time from the same plant subjected to the same conditions. Our working hypothesis involves signaling events associated with increased ABA levels, decreased glucose levels, disruption of ABA/sugar signaling, activation of programmed cell death/senescence through repression of a phospholipase C-mediated signaling pathway, and arrest of the cell cycle in the stressed ovary at 1 d after pollination. Increased invertase levels in the stressed leaf meristem, on the other hand, resulted in that tissue maintaining hexose levels at an "unstressed" level, and at lower ABA levels, which was correlated with successful resistance to drought stress.

Embryo abortion occurs when plants experience abiotic stresses such as drought or temperature extremes during their reproductive phase (Andersen et al., 2002; Feng et al., 2011; Setter et al., 2011). This effect has been reported for wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), soybean (*Glycine max*), and chickpea (*Cicer arietinum*), suggesting that this is a widespread phenomenon in the plant world (Setter et al., 2011). Maize (*Zea mays*) and rice (*Oryza sativa*) grain yield are both affected in this way by drought stress, posing a threat to food security worldwide (Boyer and McLaughlin, 2007; Ji et al., 2011). Interestingly, embryo abortion is observed even when the drought stress is relieved before fertilization has occurred (Westgate and Boyer, 1984), suggesting that the vulnerability is caused by the influence of drought on the maternal tissue, which develops extensively both before and immediately after fertilization (Andersen et al., 2002). Ovaries in tissue subjected to, or with a previous history of, drought stress stop growth within 1 to 2 d after pollination (DAP; Zinselmeier et al., 1999). Tolerance to water stress in female floral parts has been correlated with yield in maize (Setter et al., 2011). In cases where the drought stress was applied before fertilization, increased levels of reactive oxygen species (ROS) are expected to exist in the maternal tissues and to participate in other related molecular events, since this is a universal response of plant cells to an abiotic stress such as drought, as reviewed previously (Cruz de Carvalho, 2008; Suzuki et al., 2012). An altered and potentially more oxidized environment is thus created within which the embryo and endosperm begin their development.

[1] This work was supported by the National Science Foundation (Plant Genome Research Program grant no. DBI–0922747, iPlant Collaborative grant no. DBI–0735191, and grant no. ABI1062472).

[2] These authors contributed equally to the article.

[3] Present address: Metabolix, Inc., 21 Erie Street, Cambridge, MA 0219.

[4] Present address: Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544.

[5] Present address: Botswana College of Agriculture, Private Bag 0027, Gaborone, Botswana.

* Corresponding author; e-mail grene@vt.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Ruth Grene (grene@vt.edu).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.112.200444

 © 2012 American Society of Plant Biologists. All Rights Reserved.

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

The extent of delivery of photosynthetic assimilate and/or the availability of sugar substrates within the developing ovary have been correlated with the incidence of embryo abortion, and soluble and cell wall-associated invertases have been shown to be part of the hexose supply pathway to the developing embryo and endosperm, respectively (Feng et al., 2011). Expression of a soluble invertase (*lvr2*) was shown to be the most vulnerable to drought stress at 3 DAP, with cell wall-associated invertase also showing a decrease at that time (Andersen et al., 2002). The levels of the invertase substrate Suc are higher in drought-stressed than in well-watered maize ovaries from −6 to +7 DAP (Andersen et al., 2002). A set range of Suc-to-hexose ratios has been shown to be crucial for seed development (Wobus and Weber, 1999), and this ratio appears to be altered during drought stress during this early stage. Sugars function both as a nutrient source and signaling molecules in plant cells (for review, see Couée et al., 2006; Ruan et al., 2010), and ample evidence exists suggesting distinct cellular Glc and Suc signaling pathways (Bolouri-Moghaddam et al., 2010; Matiolli et al., 2011). In addition, cell wall invertase itself is likely part of a signaling pathway, since it is known to form a complex in the nucleus with PIP5K9, a component of the phosphoinositol signaling system (Lou et al., 2007). It is possible, therefore, that functions related to sugar supply, as well as those involving various sugar signaling pathways, are influenced under drought stress in developing reproductive tissue.

Water stress increases abscisic acid (ABA) levels in maize reproductive tissue, and the change has been correlated with a decreased kernel set (Setter et al., 2011). A depletion of starch in drought-stressed reproductive tissue has also been observed (Zinselmeier et al., 1999). Another manifestation of water deficit during early endosperm development (9 DAP) is decreasing endosperm cell division (Ober et al., 1991). Drought-mediated increases, specifically in endosperm ABA levels, have been shown to occur by 9 DAP and have the potential to affect many downstream processes (Ober et al., 1991; Setter and Parra, 2010). ABA-related events have been correlated with pollen sterility in wheat (Ji et al., 2011). An influence of ABA on apoplastic sugar transport (involving cell wall invertase and hexose transporters) and pollen sterility in cold-stressed rice was also observed (Oliver et al., 2007). However, no comparable effects of ABA on developing maternal tissue either before, or immediately after, pollination in maize have yet been reported.

Effects of drought on sugar signaling/metabolism, auxin- and ethylene-related processes, and trehalose metabolism have all been proposed as part of the chain of events surrounding embryo abortion in maize or rice (Yu and Setter, 2003; Boyer and McLaughlin, 2007; Jain and Khurana, 2009; Feng et al., 2011; Setter et al., 2011). Most of these studies, such as those cited above, were conducted on fertilized ovaries from 8 DAP onward, yet drought-mediated embryo abortion is observed immediately after pollination. Effects of drought on the maize transcriptome at this early, and crucial, stage of ovary development have not, as yet, been characterized, and whether the various pathways invoked in the studies enumerated above contribute to the observed cessation of embryo growth in stressed plants at as early as 1 to 2 DAP is as yet unknown. The only exceptions are the lower levels of starch and the well-established early responses of cell wall and soluble invertases to drought stress at 1 DAP, at both the transcript and enzyme activity levels, immediately after pollination (Andersen et al., 2002; Boyer and McLaughlin, 2007). The reason for the choice by most investigators of more developed drought-stressed fertilized tissue as an experimental system is a practical one; it is not possible to dissect out embryo from endosperm from maternal tissue in the ovary during the first days after fertilization. The bulk of the tissue within the kernel is maternal in origin at 1 DAP (Andersen et al., 2002).

To date, few genome-wide approaches to this phenomenon have been reported. A small complementary DNA (cDNA) microarray containing about 2,500 cDNAs from maize was used to monitor gene expression specifically in developing maize endosperm and placenta/pedicel tissues during water deficit and rewatering at 9 DAP (Yu and Setter, 2003), a time when endosperm and placental tissues could be clearly distinguished. A transcriptomic study of the effect of drought on maize ears and tassels undergoing meiosis revealed raffinose and trehalose-associated genes as well as other carbohydrate-related processes (Zhuang et al., 2007). Beyond these observations, little is understood about the chain of molecular events leading to early embryo abortion in drought-stressed maize, and virtually nothing is understood about crucial events occurring upon fertilization.

Gene expression studies in maize in response to water stress have been conducted in roots (Poroyko et al., 2007), seedlings (Zheng et al., 2004), and developing ear and tassel (Zhuang et al., 2007). Genome-wide comparisons between the drought stress responses across different developmental stages in maize have not yet been made. However, the heightened sensitivity of recently fertilized ovaries to drought stress, when compared with photosynthetic tissue, is well established (for review, see Ruan et al., 2010, and refs. therein). To advance our understanding of responses to drought stress in early embryos, we report here on the results of an RNA-Seq transcriptome analysis using Illumina deep sequencing of RNA populations obtained from well-watered and drought-stressed leaf meristem and pollinated ovaries of the fully sequenced maize (B73) genotype. We have chosen to compare the basal leaf meristem regulating vegetative leaf growth (Tardieu and Granier, 2000) with ovaries (1 DAP) to best capture gene expression events in the ovary that may lead to early embryo abortion (Boyer and McLaughlin, 2007) and to gain insights into possible causes for this specific sensitivity.

The data show that many more genes responded to drought stress in the ovary at 1 DAP than in the leaf

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

meristem. Gene Ontology (GO) enrichment analysis was carried out to reveal the biological processes differentially expressed in ovary and vegetative leaf meristem tissue. MapMan analysis (Thimm et al., 2004) supported the GO enrichment analysis for major metabolic and stress-related signaling changes. The global analysis of drought-responsive biological processes by GO enrichment and MapMan is supported by discussions of the significance of specific genes relevant to drought stress in maize and other plants. The data are discussed in the context of the susceptibility of maize kernel to drought stress and the relative robustness of meristematic vegetative tissue taken at the same time from the same plant subjected to the same conditions. A hypothesis is presented integrating the expression of metabolic, signaling, and stress-protective processes.

RESULTS

Phenotypic and Physiological Responses to Drought Stress

Maize ovaries at 1 DAP (Andersen et al., 2002) and basal leaf meristematic tissue from the three youngest leaves of maize plants at the 12-leaf stage were chosen (Tardieu and Granier, 2000) to study severe drought responses. Sets of plants were subjected to moderate and severe drought stress by withholding water. The degree of drought stress was determined by monitoring soil moisture content, relative water content, chlorophyll fluorescence (photochemical efficiency of PSII), and CO_2 gas exchange in the leaves (Table I). Soil moisture content decreased from 49.2 $cm^3\ cm^{-3}$ in well-watered controls to 21.5 $cm^3\ cm^{-3}$ for moderate drought and to 10.7 $cm^3\ cm^{-3}$ for severe drought. Relative water content consequently dropped from 97.15% (control) to 83.4% (moderate drought) and to 66.5% (severe drought). These levels of drought reduced photosynthetic rate and PSII function dramatically. Plants that had been subjected to this brief, but severe, drought stress showed significant decreases in seed set when grown to maturity (Table I). RNA isolated from plants that had been subjected to severe drought stress by withholding water for 4 d, and hence showed the phenotype of a reduced seed set when grown to maturity, was used for RNA-Seq transcriptome analysis.

RNA-Seq Analysis of Maize Drought Transcriptome Reveals Intergenic Transcripts and Novel Splice Sites

The maize drought/control RNA samples described above were used for Illumina Genome Analyzer deep sequencing. There were eight samples in total, with each condition having two biological replicates. In total, 0.3 billion reads, each 75 nucleotides long, were generated, with approximately 32 million reads from each sample. The RNA-Seq analysis workflow depicted in Figure 1 was implemented to analyze the data. The reads mapping to the genome were first categorized into three classes (Table II). Uniquely mapped reads are those that map to only one position in the genome, and splice reads are those that span across a splice junction. Multimapping reads are those that map to more than one position in the reference genome. The assembled transcripts were then classified into three main categories (Table III): known transcripts, novel isoforms of known genes, and intergenic transcripts.

Out of the 0.3 billion reads obtained by RNA-Seq, 21 million reads mapped to splice junctions, revealing 76,000 novel splice junctions that account for 23% of the total number of splice junctions identified. Of the 76,000 novel junctions, 36,000 were supported in both ovary and leaf meristem tissues, while the remaining reads were supported in either the ovary or the leaf meristem (Fig. 2). Alternative splicing events were observed in developing maize leaves by Li et al. (2010). Stress-induced alternative splicing was also evident from our data. Most of the alternatively spliced transcripts fell into very broad GO categories such as "gene expression," "nucleotide metabolic process," and "small molecule metabolic process." However, in the leaf meristem tissue, GO categories related to photosynthesis were enriched with alternatively spliced genes. Interestingly, the GO term "small GTPase-mediated signal transduction" was enriched in the ovary tissue with respect to drought-induced alternative splicing.

Table I. *Analysis of physiological parameters in maize affected by mild and severe drought at the reproductive (12-leaf) stage*

Drought was imposed 2 to 3 d before the time of hand pollination and continued for 1 DAP, at which time physiological data were obtained. Seed yield was determined at maturity. Soil moisture content (%) was calculated by the ratio of water volume to soil volume. F_v/F_m, Photochemical efficiency of PSII; ØPSII, effective quantum yield of PSII. CO_2 gas-exchange measurements were performed using LI-COR 6400XT. Plant water status was monitored by the determination of relative water content (%). Means within columns followed by different letters are significant at $P \leq 0.001$. *P* values were determined according to the LSD test.

Condition	Soil Moisture Content	Chlorophyll Fluorescence		CO_2 Exchange	Relative Water Content	Seed Number per Plant
		F_v/F_m	ØPSII			
	%			*mol* $m^{-2}\ s^{-1}$	%	
Well watered	49.2 a	0.779 a	0.71 a	29.5 a	97.15 a	139.25 a
Mild drought	21.5 b	0.7405 b	0.53 b	20.25 b	83.42 b	104.75 b
Severe drought	10.7 c	0.7212 c	0.42 c	14 c	66.5 c	34.75 c
$LSD_{(0.05)}$	5.1707	0.0171	0.0121	4.1215	4.0247	12.232
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

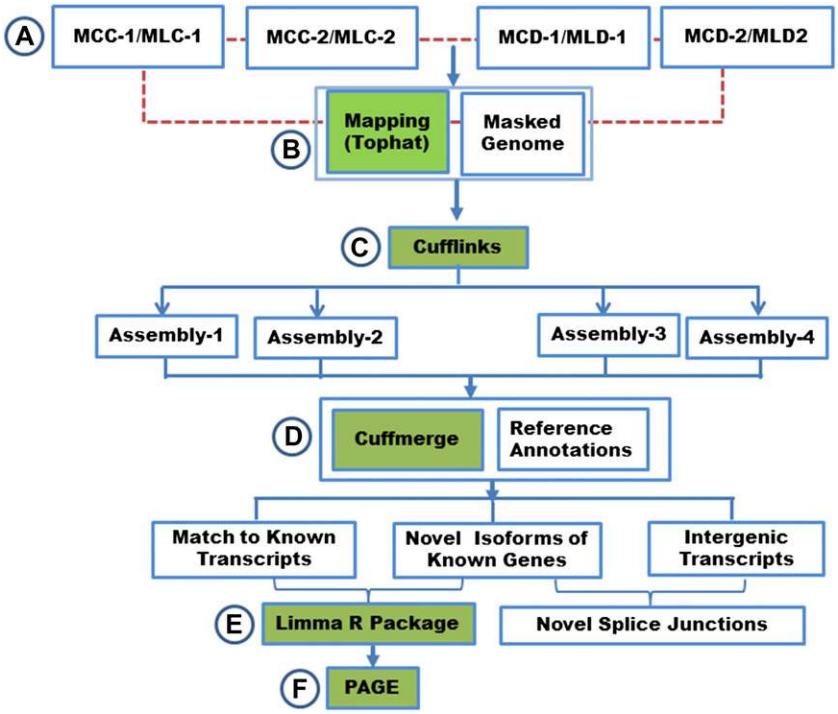


Figure 1. RNA-Seq workflow showing different steps implemented from raw reads to GO functional enrichment. A, Eight libraries were constructed. MCC and MCD stand for maize ovary tissue, well watered and drought stress, respectively; MLC and MLD stand for maize basal leaf meristem, well watered and drought stress, respectively. Numbers 1 and 2 indicate the two biological replicates. B, The reads were mapped using Tophat onto the maize masked genome. C, Cufflinks was used for transcriptome reconstruction, resulting in four different assemblies corresponding to four samples in each tissue. D, Cuffmerge was used to merge the four assemblies and the reference annotations into a single consensus list of transcripts and categorize the assembled transcripts. E, Limma, a Bioconductor R package, was used to identify differentially expressed genes under drought in both the tissues. F, Parametric analysis of gene set enrichment was implemented to identify different biological process GO terms enriched.

Differential Expression and GO Enrichment of the Maize Drought Transcriptome

Each condition was represented by two biological replicates, resulting in four pairs of samples. The transcript abundance of each gene was estimated by fragments per kilobase of exon per million fragments mapped (FPKM). Supplemental Figure S1, showing the box-plot distribution of the log FPKM values, suggests that the median and the quartile values among the samples being compared for differential expression are almost identical. To further investigate the extent of variability among the biological replicates, the coefficient of variation of FPKM values for each gene, between the replicates, was calculated. Nearly 75% of the genes, in ovaries and leaf meristem, had a coefficient of variation of less than 20% (Supplemental Fig. S2), indicating slight variability among the biological replicates. To make the calculation of differential expression more robust, since only one biological replicate was present, an R package called Limma was used (see "Materials and Methods"). After applying a very stringent cutoff, as described in "Materials and Methods," 4,382 genes with increased transcript abundance and 6,044 genes with decreased transcript abundance were identified in the maize ovary tissue; a much smaller number of genes, 462 (increased transcript abundance) and 220 (decreased transcript abundance), responded in the basal leaf meristem.

Comparable gene expression was seen between leaf meristem and ovaries in GO terms (Supplemental Table S1) related to active growth, suggesting active development and growth in both the tissues. From the heat-map representation (Fig. 3) it is also evident that

Table II. *Number of reads sequenced and mapped with Tophat*

The number of unique mapping reads plus multimapping reads equals the total number of alignments. Spliced reads are reads that map to known or novel splice junctions. MCC and MCD stand for maize ovaries, well watered and drought, respectively; MLC and MLD stand for maize basal leaf meristem, well watered and drought, respectively. Numbers 1 and 2 indicate the two biological replicates.

Sample	Sequenced Reads	Unique Mapping Reads	Spliced Reads	Multimapping Reads	Total Alignments
MCC-1	38,720,518	29,006,929	3,377,444	3,334,737	32,341,666
MCC-2	32,396,207	21,765,134	829,439	2,202,487	23,967,621
MCD-1	38,617,367	29,275,556	2,854,178	3,885,381	33,160,937
MCD-2	38,362,194	28,779,699	2,093,744	4,245,354	33,025,053
MLC-1	37,577,043	27,108,369	3,396,442	2,585,335	29,693,704
MLC-2	30,596,778	19,382,018	2,870,407	1,696,101	21,078,119
MLD-1	37,264,930	27,552,879	2,793,741	2,970,863	30,523,742
MLD-2	36,699,121	27,216,792	2,800,586	3,113,970	30,330,762

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

Table III. *Classification of transcripts produced in ovaries and basal leaf meristem under both drought and well-watered conditions*

Reads that mapped to the reference in the opposite strand of any annotated feature were considered as artifacts; this may be due to contamination with microRNA or small interfering RNA.

Class	Total Transcripts in Ovary	Assembled Reads in Ovary	Total Transcripts in Leaf Meristem	Assembled Reads in Leaf Meristem
		%		
Match to known transcripts	67.2	73.3	68.3	73.6
Novel isoforms of known genes	17.9	17.3	19.4	16.3
Intergenic	8.3	2.9	5.7	2.53
Artifacts	6.4	7.12	6.5	8.3

basal leaf meristem cells, which give rise to leaf tissue, are less affected by drought than the ovary tissue at 1 DAP. Several distinct patterns of gene expression specific to one of the two drought-stressed tissues are shown in Figure 3. GO categories corresponding to various aspects of carbon metabolism (e.g. "cellular polysaccharide metabolic process," "glucan biosynthetic process," and "organic acid biosynthetic process") were enriched with genes with decreased expression in the case of the drought-stressed ovary tissue, whereas the opposite result was observed for leaf meristem tissue. A similar contrast was observed for the GO categories "cell division" and "cell cycle." In the case of the GO categories "response to stress," "response to oxidative stress," and "oxidation-reduction process," there was no significant enrichment for the leaf meristem tissue, but the ovary tissue showed enrichment for genes with decreased transcript abundance. The category "translation" was enriched with genes with decreased transcript abundance in both tissues. For the category "vesicle docking involved in exocytosis" and several lipid-associated categories, "phosphatidylinositol metabolic process," "lipoprotein metabolic process," and "glycerolipid biosynthetic process," enrichment for genes with decreased transcript abundance was observed in the leaf meristem tissue, with no significant enrichment in the ovary tissue. A select few of these categories were chosen for detailed examination. To provide an additional level of analysis, the MapMan software tool was selected for its capacity to view statistically significant drought-mediated gene expression data for the ovaries (Fig. 4A) and the leaf meristem (Fig. 4B) tissues in the context of known metabolic pathways, biological processes, and functional categories.

Responses of Carbon Metabolism-Associated Genes to Drought

Changes in the expression levels of a large number of carbohydrate metabolic genes at 1 DAP were observed (as shown in Fig. 5, confirming earlier observations; Yu and Setter, 2003; Chen et al., 2011). Genes encoding enzymes involved in starch synthesis, namely ADP-Glc pyrophosphorylase, starch synthase, starch-branching enzyme, and starch-debranching enzymes, showed significant decreases in transcript abundance in the drought-stressed ovary (Fig. 5A), whereas no changes were observed in the drought-stressed leaf meristem. A large subunit of ADP-Glc pyrophosphorylase (GRMZM2G391936), the first committed step in starch synthesis, showed decreased transcript abundance in the ovary. Another AGPase gene, here named *Glc 1-phosphate adenyl transferase* (GRMZM2G144002), also showed decreased expression in the drought-stressed ovary tissue. *Granule-bound starch synthaseI* (*GBSSI*; GRMZM2G024993) and *GBSSIIa* precursor also showed decreased expression in the drought-stressed ovary tissue. In addition to the GBSS family, several isoforms of soluble starch synthases and starch-branching enzymes showed decreased expression in the drought-stressed ovary tissue (Fig. 5A, bins 2 and 3); however, *starch synthaseV* (GRMZM2G130043) showed increased transcript abundance. On the other hand, a large proportion of the genes encoding α- and β-amylase isoforms (Fig. 5A, bin 5), which function in starch breakdown, showed increased expression in the drought-stressed ovary tissue.

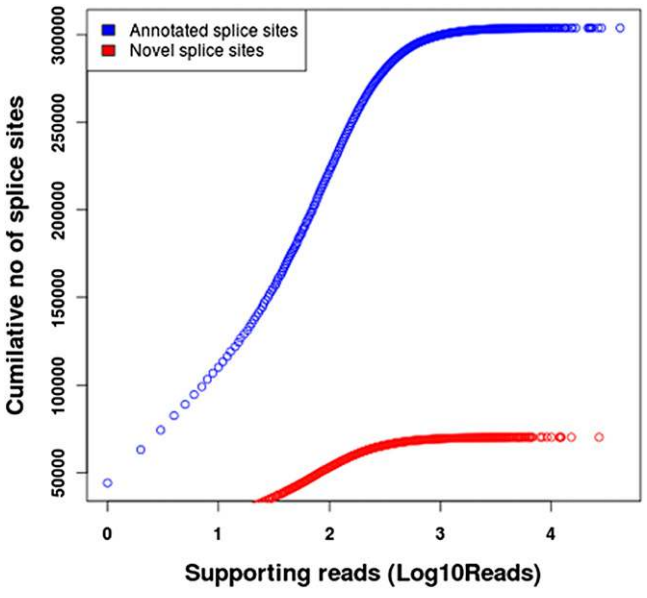


Figure 2. Relationship of the cumulative number of splice junctions of each type to the log of the number of supporting reads. All the splice junctions identified were classified as either annotated (present in the gene model) or novel (absent in the gene model).

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

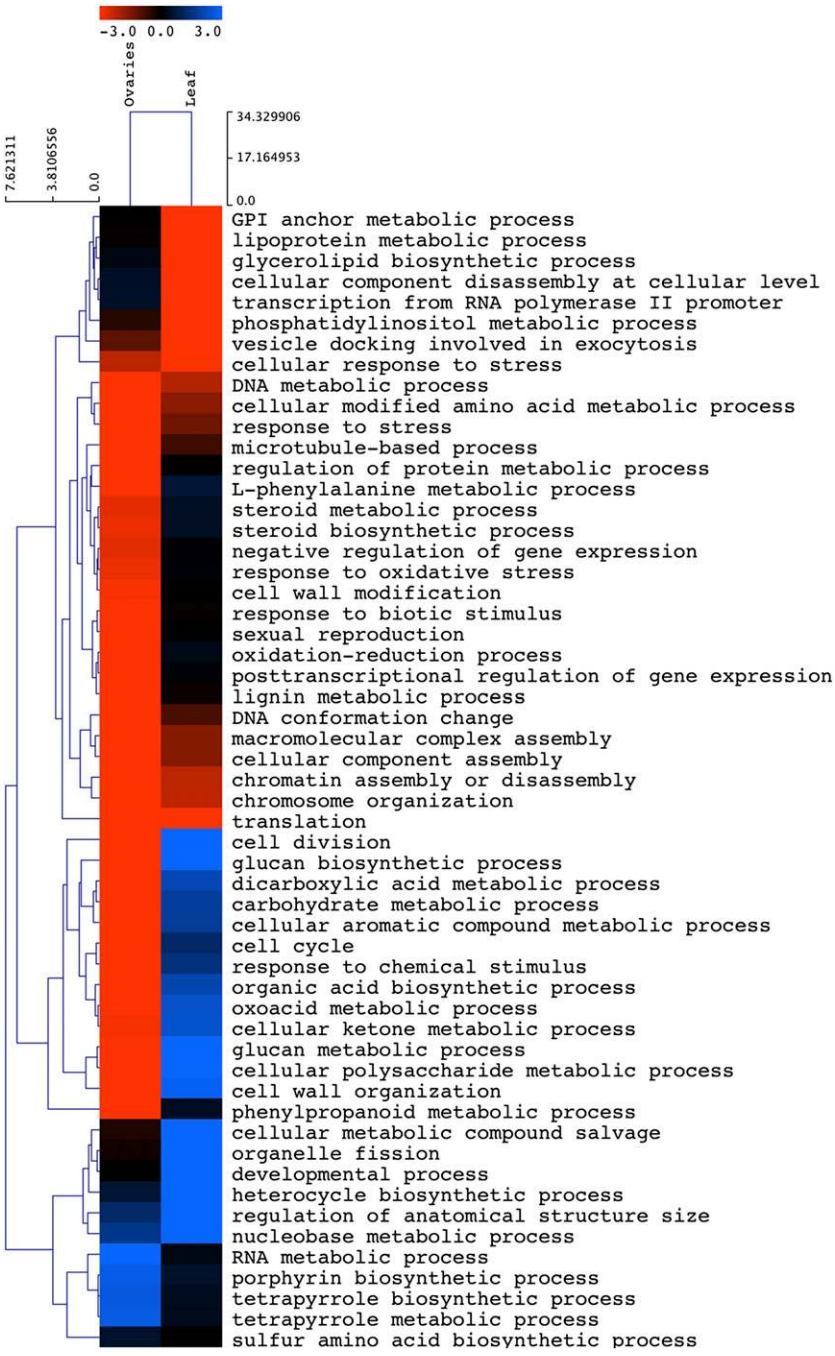


Figure 3. Heat map showing the different biological process GO terms enriched in maize reproductive and vegetative tissue under drought stress. The *T* statistic obtained from the Limma R package was used as a parameter for the parametric analysis of gene set enrichment analysis. A *q* value cutoff of 0.05 was used to select enriched GO terms in both the tissues. The heat map shows the Z score as obtained from parametric analysis of gene set enrichment for terms enriched in either ovary tissue or leaf meristem.

Two genes encoding Suc synthase (GRMZM2G089713 and GRMZM2G318780) showed increased transcript abundance in the ovary tissue. Another gene encoding Suc synthase2 (GRMZM2G152908) showed decreased transcript abundance. In contrast to the results of Kopka et al. (1997) obtained from drought-stressed potato (*Solanum tuberosum*) leaves and guard cells, no drought-mediated changes in transcript abundance of genes associated with Suc or starch metabolism were observed in maize leaf meristem. However, there was one exception. A drought-mediated increase in the expression of a vacuolar invertase was observed in the maize leaf meristem (see below). In addition, genes encoding fructokinase2 (GRMZM2G051677) and hexokinase1 (HXK1; GRMZM2G432801), which function in a signaling role (Cho et al., 2010) as well as an enzymatic role, showed significant decreases in expression in the drought-stressed ovary. In its enzymatic capacity, hexokinase action leads to hexoses entering the glycolytic pathway. Interestingly, another isoform of *hexokinase1* (GRMZM2G051806) was not affected in the ovaries but showed increased expression in the

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

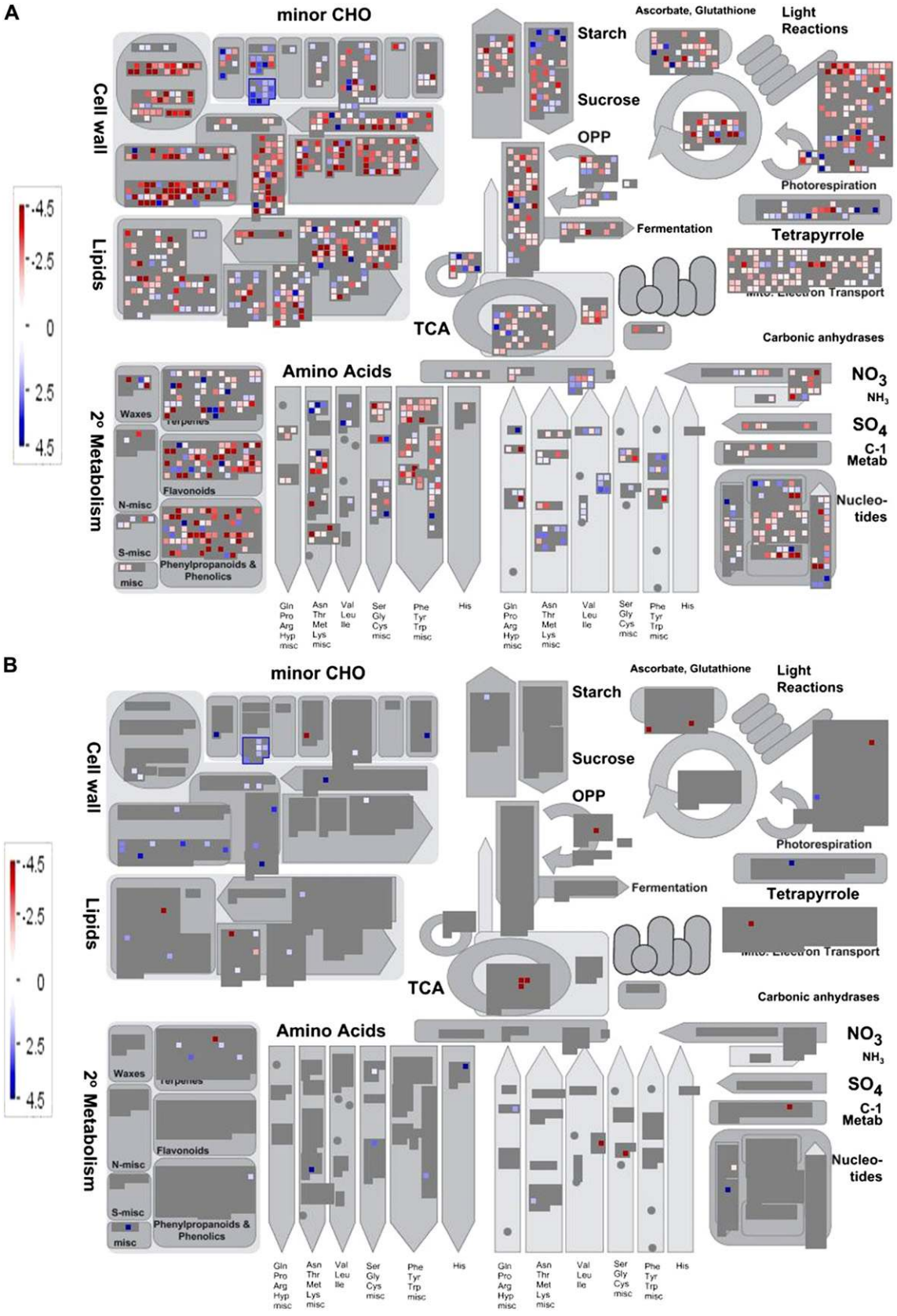
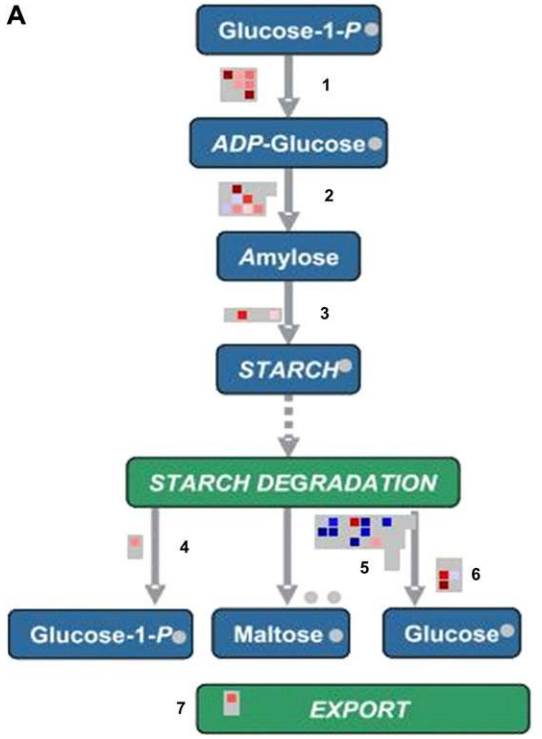


Figure 4. Overview of differentially expressed transcripts involved in different metabolic processes under drought stress. A and B show drought-mediated expression changes in different metabolic processes in the ovary and leaf meristem, respectively. The images were obtained using MapMan, showing different functional categories that passed the cutoff (less than 0.05 *q* value and greater than 2-fold change) for differential expression.

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022



Bin ID	Maize ID	Annotation	Ovaries (fold Change)
1	GRMZM2G144002	Glucose-1-phosphate adenylyltransferase large subunit 3 precursor	11.11
	GRMZM2G429899	Glucose-1-phosphate adenylyltransferase large subunit 1	2.86
	GRMZM2G027955	Glucose-1-phosphate adenylyltransferase large subunit 2	2.94
	GRMZM2G163437	Glucose-1-phosphate adenylyltransferase small subunit fragment	3.70
	GRMZM2G068506	Similar to ADP glucose pyrophosphorylase 1	3.33
	GRMZM2G391936	Plastid ADP-glucose pyrophosphorylase large subunit	33.33
2	GRMZM2G008263	Granule bound starch synthase IIa precursor	14.29
	GRMZM2G130043	Starch synthase V	2.10
	GRMZM2G348551	Similar to starch synthase 2	4.55
	GRMZM2G024993	Granule-bound starch synthase 1	2.17
	GRMZM2G141399	Starch synthase DULL1	3.33
3	GRMZM2G032628	1,4-alpha-glucan-branching enzyme 2	5.00
	GRMZM2G073054	Starch branching enzyme IIa fragment	2.17
4	GRMZM2G074158	Phosphorylase	3.13
5	GRMZM2G081502	Similar to alpha-amylase	13.53
	GRMZM2G025833	Beta-amylase	26.10
	GRMZM2G103055	Alpha-amylase	30.48
	GRMZM2G074781	Similar to alpha-amylase	13.30
	GRMZM2G138468	Alpha-amylase	6.02
	GRMZM2G058310	Beta-amylase	2.94
	GRMZM2G082034	Beta-amylase	6.13
6	GRMZM2G136803	Similar to disproportionating enzyme 2	5.88
	GRMZM2G013811	Similar to disproportionating enzyme 2	8.33
7	GRMZM2G156356	Maltose excess protein 1-like	4.00
8	GRMZM2G008507	Similar to sucrose-phosphate synthase family protein	4.55
9	GRMZM2G083248	Similar to sucrose transporter 2	5.13
	GRMZM2G081589	Similar to sucrose transporter 2	8.33
	GRMZM2G034302	sucrose transporter1	9.08
10	GRMZM2G089713	Sucrose synthase 1	3.39
	GRMZM2G152908	Sucrose synthase 2	8.33
	GRMZM2G318780	sucrose synthase2	2.85
11	GRMZM2G115451	Plant neutral invertase family protein	7.69
	GRMZM2G477236	Similar to light harvesting like protein Lil3	2.78
	GRMZM2G007277	Similar to plant neutral invertase family protein	6.25
	GRMZM2G118737	Similar to plant neutral invertase family protein	2.04
	GRMZM2G119689	Similar to cell wall invertase 2	12.50
	GRMZM2G174249	Siimilar to cell wall invertase 4	4.17
	GRMZM2G089836	Beta-fructofuranosidase 1	2.27
	GRMZM2G394450	Beta-fructofuranosidase 1 Precursor	10.00
12	GRMZM2G051677	Fructokinase-2	7.69
	GRMZM2G072091	Similar to pfkB-like carbohydrate kinase family protein	8.33
	GRMZM2G466780	protein kinase	3.81
13	GRMZM2G104081	Similar to hexokinase 1	5.35
	GRMZM2G432801	Hexokinase-1	3.13

Figure 5. Effects of drought stress on the expression of genes associated with starch and Suc metabolism. Drought stress responses associated with starch (A) and Suc metabolism (B) in ovaries are shown, with a table showing gene names, putative functions, and fold change values. The values in red and blue indicate fold increase and decrease in expression in the drought-stressed tissue, respectively.

drought-stressed leaf meristem. Thus, sugar signaling may have responded to drought stress in both tissues, but in opposite directions.

Two genes (GRMZM5G872256 and GRMZM2G165919) encoding galactinol synthase showed increased expression in the drought-stressed ovary tissue, whereas no galactinol synthase genes were differentially expressed under drought stress in the leaf meristem (Supplemental Table S2). Three genes (GRMZM2G077181, GRMZM2G150906, and GRMZM2G340656) annotated as "raffinose synthase" showed increased expression, while two genes (GRMZM2G311756 and GRMZM2G047292) showed decreased expression in the drought-stressed ovary tissue (Supplemental Table S2). Only one raffinose synthase gene (GRMZM2G340656) showed increased transcript abundance in the leaf meristem. Two trehalose phosphate synthase (TPS) genes and several putative TPS genes showed increased expression in the ovary tissue, while some putative TPS genes showed increased expression in the leaf meristem (Supplemental Table S3).

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

Three trehalose phosphate phosphatase genes showed decreased expression, and five of them showed increased expression in the drought-stressed ovary tissue; none of them were differentially expressed in the leaf meristem. Thus, two carbohydrate pathways associated with responses to abiotic stress in plants responded in both tissues.

Overall, these data suggest a down-regulation of starch and Suc biosynthesis and an up-regulation of starch and Suc degradation in the drought-stressed ovary, with no comparable change in the drought-stressed leaf meristem. The data also suggest possible increased activity of raffinose- and trehalose-associated metabolism in both tissues.

Cell Cycle and Division Genes Show Contrasting Expression Patterns in Vegetative and Reproductive Tissues

The maize expression data annotated by the GO category "cell cycle" (GO:0007049) consists of a group of 49 genes with an ovary tissue Z score of −3.603 and a leaf meristem tissue Z score of 1.221 (Fig. 6). Genes that did not pass the cutoff as mentioned in "Materials and Methods" were excluded from further analysis. The identity of the gene product was assigned from existing maize annotations or from homologous rice or Arabidopsis (*Arabidopsis thaliana*) annotations in cases where the maize annotation was ambiguous.

Eleven cyclins were identified: nine of them showed decreased expression and one, *CYCLIN D4* (GRMZM2G075117), showed increased expression in the drought-stressed ovary tissue (Fig. 6). Two genes (GRMZM2G140633 and GRMZM2G476685) with the same cyclin δ-2 annotation showed increased expression in the leaf meristem tissue (Supplemental Table S4). The transcript levels of two genes annotated as cyclin-dependent kinase inhibitor (CDKI) were increased in the ovary tissue (GRMZM2G116885 and GRMZM2G157510), and one displayed increased transcript abundance in the leaf meristem tissue (GRMZM2G157510). Six other cell cycle-associated genes (*Rad17*, *Rad51*, *MAD2*, *Nuf2*, *ZmRBR3*, and *ROA1*) exhibited decreased transcript

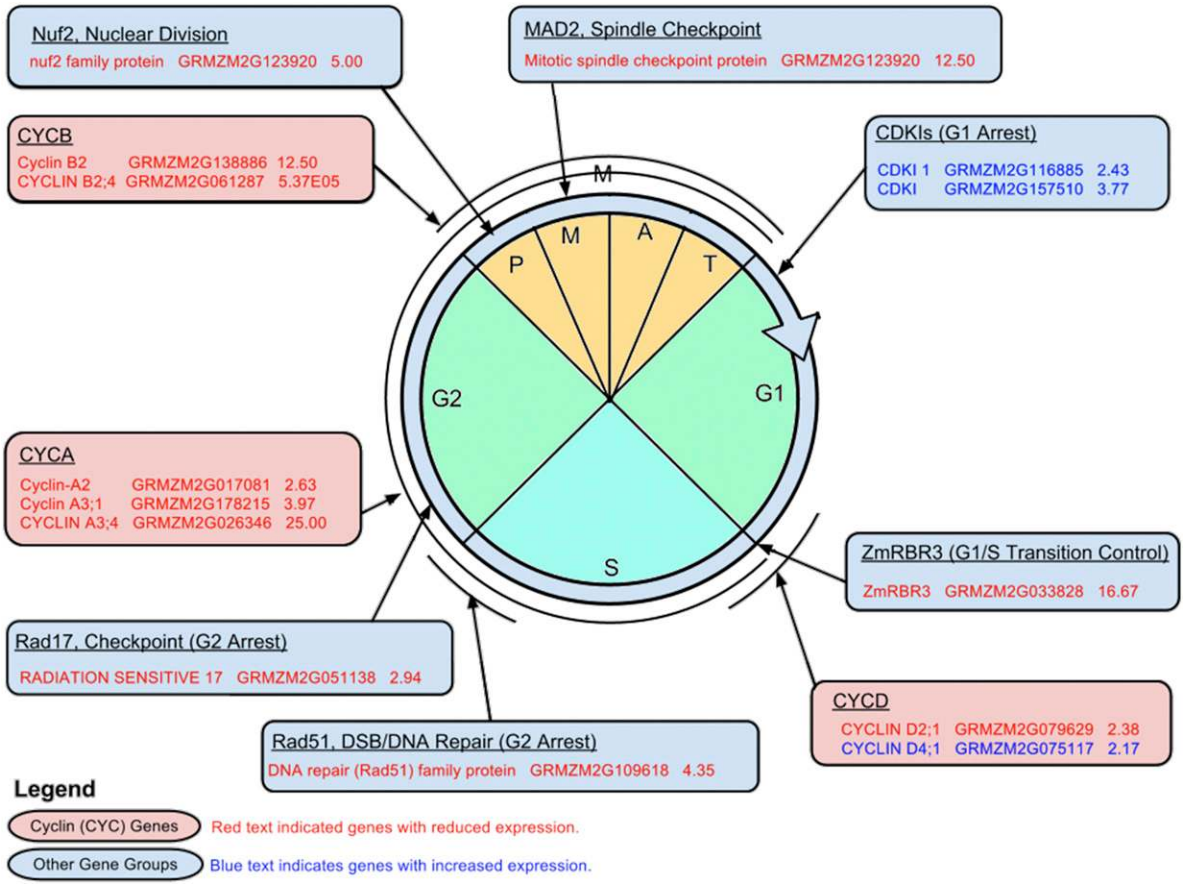


Figure 6. RNA-Seq expression data in drought-stressed ovary tissue with associated cell cycle phases. Each box represents a gene product, or group of gene products, and indicates where they exert influence on the cell cycle. Cyclin (CYC) gene group boxes display a red background, and other cell cycle gene groups have a light blue background. Within each box, the expression data for drought-stressed ovary tissue is listed in the form annotation, maize gene identifier, and fold change. The values in red and blue indicate the fold decrease and increase in expression in the drought-stressed ovary tissue, respectively.

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

abundance in the ovary tissue, with *MAD2* exhibiting increased transcript abundance in the leaf meristem tissue (GRMZM2G047143). *ZmRBR1* and *ZmRBR2* showed increased transcript abundance but did not make the significance cutoff. Supplemental Table S4 summarizes the tissue-specific gene expression responses with their accompanying cell cycle classification. The effect of drought on the expression of genes in the ovary is associated with the cell cycle phases as shown in Figure 6.

Programmed Cell Death Genes Display Contrasting Expression Patterns in Vegetative and Reproductive Tissue

The annotations of the RNA-Seq data were searched for known programmed cell death (PCD) gene terms from animal, yeast, and plant. Twenty-six genes were identified in the data set with matches across maize, rice, or Arabidopsis annotations. Of the 26 matches, 12 were identified with significant expression changes above the cutoff as mentioned in "Materials and Methods." *Metacaspase5* (GRMZM2G066041), *MLO Family homolog4* (GRMZM2G089259), and *BAX inhibitor1* (GRMZM2G029087) showed increased transcript abundance in drought-stressed leaf meristem tissue; conversely, none of the genes showed decreased transcript abundance. Of the 11 significant genes in ovary tissue, nine genes (*LSD1*, two metacaspases, five MLO family members, and BAX inhibitor) showed decreased transcript abundance and two genes (*LOL3* and *Metacaspase1*) showed increased transcript abundance (for summation, see Supplemental Table S5).

Responses of Antioxidant and ABA-Related Genes

In the ovary tissue, 42 genes in the GO category "response to oxidative stress" were significantly affected by severe drought (Supplemental Table S6). Of these, all but six showed decreased transcript abundance; homologs of catalase2 and APX4 were among the genes that showed increased transcript abundance. In contrast, only one gene in this GO category, belonging to the peroxidase family, responded to drought stress in the leaf meristem, and it showed decreased transcript abundance. The transcript abundance of a chalcone synthase gene belonging to this category was dramatically decreased in the ovary. The majority of genes that showed decreased transcript abundance in the ovary were in the peroxidase superfamily class. Homologs of several members of the ascorbate peroxidase group, such as APX1, showed decreased transcript abundance. Genes for thioredoxin reductase, a glutathione-*S*-transferase, a cytosolic copper/zinc, and a mitochondrial manganese-superoxide dismutase showed decreased transcript abundance. It appears from these data that the drought-stressed ovary tissue may not have been adequately protected from ROS attack because of the decreased expression of these antioxidant genes in the drought-stressed ovary.

Three genes (GRMZM2G407181, GRMZM2G110192, and GRMZM5G858784) belonging to the 9-cis-epoxycarotenoid dioxygenase (NCED) gene family, encoding enzymes of the ABA biosynthetic pathway, showed increased transcript abundance in the ovary tissue (Supplemental Table S7). Only one NCED gene (GRMZM2G417954) that was not expressed in the ovary showed increased transcript abundance by drought in the leaf meristem. Two genes (GRMZM2G127139 and GRMZM2G136344) annotated to the zeaxanthin epoxidase gene family showed increased transcript abundance in the ovary tissue; neither of them were differentially expressed in the leaf meristem under drought stress. In contrast, three of the genes (GRMZM2G124175, GRMZM2G141535, and GRMZM2G058037) that showed decreased transcript abundance in the ovary were the genes belonging to the aldehyde oxidase gene family, and none of them were differentially expressed in the leaf meristem. Two genes, GRMZM2G168079 and GRMZM2G479760, which have weak similarity to *ABA insensitive5* (*ABI5*) and *ABA binding factor4* (*ABF4*) in Arabidopsis, showed increased transcript abundance in the ovary tissue. Many GRAM domain-containing proteins, known to be responsive to ABA (Jiang et al., 2008), showed increased transcript abundance, which is consistent with the increase in the expression of ABA biosynthetic genes in the ovary discussed above. It appears that ABA biosynthesis was stimulated by exposure to severe drought stress in both tissues. ABA signaling genes, on the other hand, were responsive to drought in the ovaries only.

Sugar, Phosphatidylinositol Signaling-Associated, and Phospholipase-Related Responses

The responses to drought among genes known to respond either positively or negatively to ABA and/or Glc and those that are documented to be regulated by HXK1 are shown in Table IV. Overall, genes whose expression is positively regulated by Glc show a decrease in expression in the drought-stressed ovaries, whereas there were no significant responses in this category in the leaf meristem. In contrast, there were mixed responses among phosphatidylinositol (PI) signaling-related genes.

Seventeen genes annotated to phospholipase C (PLC) were identified in the expression data set. In Supplemental Table S5, the matches are summarized by broad categories under phospholipase A, PLC, phospholipase D (PLD), and miscellaneous. Of the four PLCs, three (GRMZM2G139041, GRMZM2G081719, and GRMZM2G137435) showed significant increases in transcript abundance in the ovary tissue and insignificant changes in the leaf meristem. *Phosphatidylinositol-specific phospholipaseC4* (GRMZM2G114354) showed an insignificant change in expression in the ovary and a massive decrease in transcript abundance in the leaf meristem. Of the five PLDs, three (GRMZM2G054559, GRMZM2G442551, and GRMZM2G108912) exhibited decreases in transcript abundance in the ovary and two

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

Table IV. *Effects of drought stress on the expression of genes associated with energy level signaling in ovary and leaf meristem tissue*

Annotation sources are as follows: maize (Zm; MaizeSequence.org), rice (Os; Rice Genome Annotation Project), Arabidopsis (At; The Arabidopsis Information Resource). The values in boldface and italic type indicate the fold decrease and increase in expression in the drought-stressed tissue, respectively. Dashes indicate failure to meet the significance cutoff or undetected. X indicates a known positive regulation or known hexokinase-mediated signaling. Vertical bars represent "either/or". HXK Dep., Hexokinase dependent activity.

Gene	Glu (+)	Glu (−)	ABA (+)	ABA (−)	HXK Dep.	Annotation	Ovary	Leaf	References
							fold change		
GRMZM2G119941	X					Invertase cell wall4, mRNA$^{(At)}$	–	*2.55*	Ruan et al. (2010)
GRMZM2G174249	X					gpm24 | hypothetical protein LOC100279267 (in At cell wall invertase4)$^{(At)}$	**4.17**	–	Ruan et al. (2010)
GRMZM2G119689	X					Miniature seed1 (in At cell wall invertase2)$^{(At)}$	**12.5**	–	Vilhar et al. (2002)
GRMZM2G422750	X					c2 | chalcone synthaseC2 (naringenin-chalcone synthaseC2)$^{(Zm)}$	**2.3E+08**	–	Xiao et al. (2000)
GRMZM2G104081		X	X			Hexokinase1$^{(At)}$	*5.35*	–	Cho et al. (2006)
GRMZM2G477236	X				X	Similar to chlorophyll *a*/*b*-binding family protein (AT4G17600)	**2.78**	–	Cho et al. (2010)
GRMZM2G391936	X				X	Large subunit of AGPase, homologous to APL3$^{(At)}$	**33.33**	–	Li et al. (2006)
GRMZM2G048085		X			X	Senescence-associated protein DIN1	*4.63*	–	Valluru and Van den Ende (2011)
GRMZM2G077278		X			X	SNF1 kinase homolog10$^{(At)}$	*2.2*	–	Hanson and Smeekens (2009)
GRMZM2G168079		X	X		X	ABI5 | ABA response element-binding factor (LOC100285149), mRNA$^{(Zm)}$	*13.64*	–	Arroyo et al. (2003)

(GRMZM2G019029 and GRMZM2G179792) showed increased transcript abundance in the ovary. With the exception of GRMZM2G019029, all PLDs displayed no significant changes in expression level in the leaf meristem. The three PLDα1 genes showed mixed expression, with two of them showing increased and one showing decreased transcript abundance in the ovary.

Quantitative Real-Time-PCR Validation of Differentially Expressed Transcripts from RNA-Seq

To confirm the accuracy and reproducibility of the Illumina RNA-Seq results, a few genes that showed increased transcript abundance, decreased transcript abundance, and nondifferentially expressed genes under drought stress were chosen for quantitative real-time (qRT)-PCR (Fig. 7A). Correlation between RNA-Seq and real-time PCR was evaluated using fold change measurements. For comparison of fold change, scatterplots were generated using the $\log_2$ fold change determined between RNA-Seq and qRT-PCR, which is defined as $\Delta\Delta C_T$ (for comparative threshold cycle). As shown in Figure 7A, the qRT-PCR results revealed that the expression trends of these genes showed significant similarity ($r^2 = 0.88$) with the RNA-Seq data.

Quantitative Expression Analysis of Maize Vacuolar, Cell Wall, and Neutral Invertases

To compare previous observations, and to extend those observations to more members of the invertase family, drought-mediated relative expression levels of eight members of the invertase gene family in both tissues were measured using qRT-PCR. As shown in Figure 7B, the expression of two soluble (*Inv1* and *Inv2*), three cell wall (*Incw1*, *Incw2*, and *Incw4*), and three neutral (*Inn1*, *Inn2*, and *Inn3*) invertases showed significant decreases in transcript abundance under drought in the ovary tissue. In contrast, one soluble invertase (*Inv1*) showed drought-mediated increased transcript abundance in the leaf meristem tissue. *Incw1* and *Inn2* also showed increased transcript abundance in the leaf meristem, but the change was not statistically significant. Primers used to amplify the above invertase class of genes are shown in Supplemental Table S9.

DISCUSSION

The analysis of the maize drought transcriptome by RNA-Seq led to approximately 73% of the assembled reads in both tissues being matched to known transcripts as predicted by genome sequencing and annotation (Table III). The remaining transcripts comprise 16% to 17% novel spliced isoforms and 2.5% to 3% intergenic transcripts. Out of the known transcripts, 13,709 and 3,942 transcripts in the ovary and leaf meristem, respectively, are drought responsive. Among the novel isoforms identified, 4,174 in the ovaries and 2,404 in the leaf meristem are drought responsive. The alternatively spliced isoforms representing approximately 22% of total assembled reads suggest a relatively high level of drought stress-

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

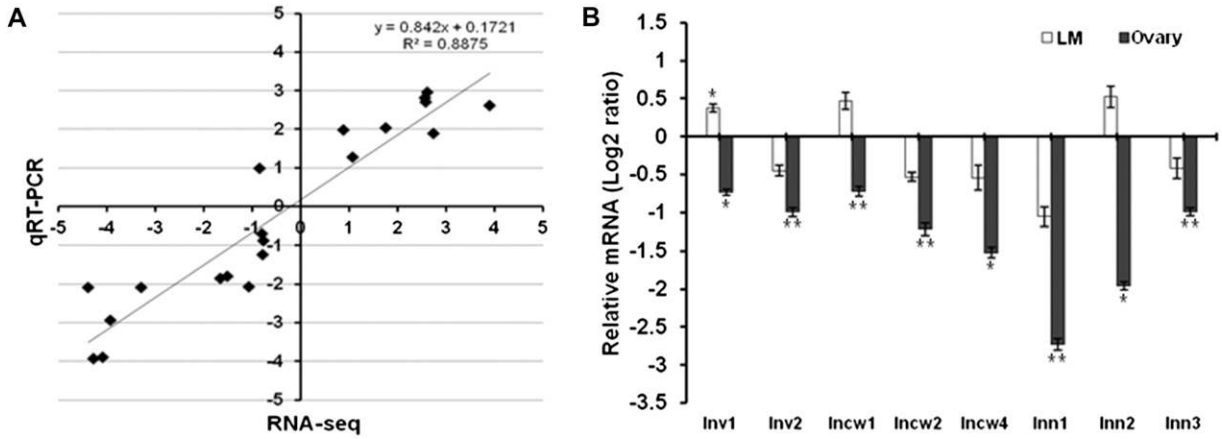


Figure 7. qRT-PCR validation of differentially expressed genes in maize under drought. A, Correlation of fold change analyzed by RNA-Seq platform (*x* axis) with data obtained using real-time PCR (*y* axis). B, Expression analysis of maize vacuolar, cell wall, and neutral invertases assessed through qRT-PCR. Error bars represent SE ($n = 3$). Asterisks indicate levels of significance of differential expression (*t* test: * $P \leq 0.05$, ** $P \leq 0.01$).

responsive posttranscriptional regulation. This is in agreement with a recent whole-genome-wide transcriptional analysis where environmental/stress responses were manifested in posttranscriptional events, often leading to changes in RNA stability (Staiger and Green, 2011).

Embryo development in cereals is especially sensitive to drought stress. The most drastic effect of drought stress on fertilized maize ovaries is known to occur at the earliest period in embryo development and likely involves stress responses that affect differentiation processes specific to the early developing seed. The mechanisms underlying this phenomenon are not understood, and high-throughput studies of drought-stressed reproductive tissue of maize have not been available until now. The results of deep sequencing of drought-stressed maize reproductive tissue 1 DAP and comparison with the transcriptome of leaf meristematic tissue are presented here. The data reveal effects of drought stress on transcript abundance that occur in (either or both) ovaries 1 DAP and leaf meristematic tissue. The multiplicity of GO categories found to be enriched in drought-stressed tissues (Fig. 3) speaks to the complexity of the response. The quantitative PCR analysis served as a validation of the RNA-Seq data (Fig. 7A), which could be described in greater depth.

Carbohydrate-Related Responses: Overview

Several key enzymes in the metabolic pathways that direct carbon flow and process assimilates were differentially expressed in the two maize tissues under drought stress (Fig. 4). The gene expression data are consistent with a drought-mediated decrease in starch biosynthesis and an increase in starch and Suc breakdown in the ovaries. With the exception of an insoluble invertase, no significant change in the expression of starch or Suc-related genes was detected in the leaf meristem tissue. This is in contrast with the results obtained by Kopka et al. (1997) in potato. Several genes known to be involved in Suc processing were differentially expressed under drought, confirming earlier observations (Boyer and McLaughlin, 2007). The data are also compatible with an increase in biosynthetic activity of the raffinose oligosaccharide pathway and with increased trehalose metabolism, each in the drought-stressed ovaries only.

Starch and Suc Metabolism

Genes encoding starch biosynthesis enzymes showed decreased transcript abundance and those encoding starch degradation showed increased transcript abundance in the drought-stressed ovaries; however, there was no response in the leaf meristem among those categories. These results validate the findings of Zinselmeier et al. (1999), who observed a lower level of starch in water-stressed ovaries. In cereals, GBSSI is encoded by the *Waxy* (*Wx*) locus, and the role of *GBSSI* is mostly confined to storage tissues such as the seed endosperm. GBSS had an important effect on amylose synthesis and is exclusively bound to the starch granule (Yan et al., 2009). Wx protein, which is produced by the *Wx* gene, is a GBSS responsible for amylose synthesis in cereal endosperm and pollen. Maize endosperm contains at least five starch synthase isoforms that are categorized according to conserved sequence relationships (Cao et al., 2000). In our study, we observed decreased transcript abundance of multiple starch synthase isoforms (Fig. 5A). Starch synthase isoforms have unique roles in starch synthesis, and this has been experimentally validated through mutant studies in different plants. For example, SSIIIa mutants of rice (Fujita et al., 2007) and maize (Gao et al., 1998) are deficient in long chains of amylopectin, suggesting their specific role in chain elongation.

It is interesting to find that genes encoding several steps in starch biosynthesis showed decreased transcript

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

abundance, and not simply subunits of AGPase, suggesting a mechanism that coordinates this effect. Other effects of repressing AGPase expression were reported by Weigelt et al. (2009) in AGPase knockdown pea (*Pisum sativum*) seeds. Seeds of the knockdown plants showed an expected metabolic shift in composition from starch toward the accumulation of sugars but also the repression of cell wall and vacuolar invertase gene expression, an increase in Suc synthase expression, increases in ROS, and changes in the interactions of ABA and sugar signaling. These changes resonate with our data on coordinated regulation, although the pea seeds studied by Weigelt et al. (2009) were quite mature compared with the maize ovaries, and no imposed abiotic stress was involved.

Several isoforms of α- and β-amylases showed increased transcript abundance in the stressed ovaries (Fig. 5A, bin 5). The breakdown of starch in plant storage organs is catalyzed by amylase, this being the only enzyme known to attack raw starch granules (Kaplan and Guy, 2004). The cooperative action of a range of other degrading enzymes, including β-amylase, debranching enzyme, and starch phosphorylase, results in the dissolution of the stored starch, leading to the production of a number of oligosaccharides, and finally to maltose, Glc, or Glc-1-P, as shown in Figure 5A. The gene encoding maltose excess protein1-like (GRMZM2G156356) showed decreased transcript abundance at 1 DAP (Fig. 5A). The increase in the transcript levels of the two Suc synthases suggests the possibility of Suc hydrolysis and the entrance of hexose as the primary sugar in endosperm metabolism. However, since low Glc signaling, which is a response to carbon deprivation, appears to have been activated (Table IV), not every tissue or cell type in the ovary at 1 DAP is likely to have received sufficient hexose to meet its metabolic needs.

Invertase in Its Metabolic and Likely Signaling Role

Invertase activity plays a metabolic role during early kernel development by converting Suc originating from source leaves into hexoses to support cell division in the endosperm and embryo (Bate et al., 2004). RNA-Seq deep sequencing analysis revealed that the expression levels of several members of the invertase gene family decreased. As the activities of invertases are critical in maize at early stages of pollination, we sought to reliably quantify the expression changes through qRT-PCR for the individual genes of the invertase family for the two different tissues. As shown in Figure 7B, the expression of the genes for two soluble invertase (*Ivr1* and *Ivr2*), three cell wall invertases (*Incw1*, *Incw2*, and *Incw4*), and three neutral invertases (*Inn1*, *Inn2*, and *Inn3*) showed significant decreases in transcript abundance by drought in immature reproductive tissue. On the other hand, one of the invertases (*Inv1*) showed a slight increase in transcript abundance in the leaf meristem. Additionally, as revealed by RNA-Seq analysis, three of the genes for proteins similar to neutral invertases (GRMZM2G007277, GRMZM2G115451, and GRMZM2G18737) showed significant decreases in transcript abundance. It has been widely reported that invertase activity in the developing seeds of maize is mainly due to two cell wall invertase genes (Chourey et al., 2006). In a mutant deficient in *INCW2*, reduced cell number and cell size were also observed (Vilhar et al., 2002). An equivalent result was observed in our data, where the expression of *Incw1* and *Incw2* is lowered (Fig. 7B), the cell cycle appears to be inhibited (Fig. 6), and ultimately seed number is reduced (Table I).

Both cell wall and soluble invertases showed decreased transcript abundance under drought stress in the ovary, whereas one soluble invertase showed increased transcript abundance in the leaf meristem. This suggests a distinct difference in the response of sugar metabolism, and most likely sugar signaling, between the two tissues under drought stress. The decrease in transcript abundance of invertases in the ovary echoes the results of Andersen et al. (2002), who also reported an accompanying buildup of Suc in that tissue under drought stress. The consequence of this is most likely a decrease in Glc levels, one of the products of invertase activity, disrupting the hexose-Suc ratios in the ovary, as discussed by Andersen et al. (2002). Furthermore, the buildup of Suc in drought-stressed ovaries demonstrated by Andersen et al. (2002) makes it less likely that the supply of photoassimilate to the ovary is a crucial player in the early embryo abortion phenomenon, as opposed to later stages of seed development, when demand for carbon skeletons is much greater.

Cell wall invertases may also play a signaling role since, in Arabidopsis, a cell wall invertase was demonstrated to bind to phosphatidyl monophosphate 5-kinase, a component of a signaling pathway associated with the regulation of growth and the mediation of cellular drought signaling (Lou et al., 2007; Ruan et al., 2010). However, PLC genes, which catalyze the crucial IP3-enerating step in PI signaling (Gillaspy, 2011), showed decreases in transcript abundance in both tissues, suggesting that differences in PI signaling may not form part of the mechanism(s) that underlies the relative sensitivity of early maize embryos to drought stress.

The result of the repression of the soluble invertase will be less substrate available to associate with mitochondrial hexokinase to provide ADP to maintain coupled electron transport (Xiang et al., 2011). Hexokinase, as a signaling molecule, senses a decrease in available Glc and activates the SnRK1 pathway; however, in the drought-stressed ovaries, hexokinase most likely cannot function metabolically because Glc is not available. Under these conditions, increased ROS arise in the mitochondrion and, most likely, other subcellular locations (due to a lack of substrate) to the point where the ROS-scavenging genes are repressed, a symptom of stress susceptibility. Comparable events were not detected in the leaf meristem; in contrast, in the leaf meristem, the increase in invertase activity can be interpreted as a response to a decrease in available carbon skeletons.

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

HXK1 in Its Metabolic and Signaling Role

HXK1 exerts the same catalytic function as other members of the gene family. A continuous and efficient supply of Glc to hexokinases, including the enzyme that is associated with the mitochondrion, is necessary to maintain its activity at a rather constant level, which is needed to control the flux through the mitochondrial electron transport chain and avoid ROS buildup (Camacho-Pereira et al., 2009; Xiang et al., 2011).

The maize homolog of *HXK1*, the sensor of low Glc levels (Hanson and Smeekens, 2009), showed increased transcript abundance in the ovary, as did HXK1 targets such as a homolog of ABI5, while a homolog of CAB2 showed decreased transcript abundance, as predicted by the HXK1-sensing pathway (Table IV). This is consistent with signaling through the low-Glc-signaling pathway established in Arabidopsis (Cho et al., 2006; Baena-González et al., 2007; Xue et al., 2008; Valluru and Van den Ende, 2011). The response of the CAB homolog in a nonphotosynthetic tissue, such as the maize ovary, suggests that this gene can function as a signaling component in an environment in which the encoded protein is not fulfilling its better known role as part of the photosynthetic apparatus.

The low-Glc-sensing pathway functions under stress conditions, and under carbon deprivation with low Glc it is reported that HXK1 signaling is uncoupled from stress responses and from its interaction with ABA-related events (Cho et al., 2006). This may have occurred in the drought-stressed maize ovaries. However, since the experimental system employed by those workers is so far removed from the maize system, it is difficult to determine whether such an uncoupling took place under drought in the drought-stressed ovaries.

Stress Responses in Raffinose and Trehalose Metabolism

Genes annotated to galactinol synthase and "seed imbibition proteins" are known to be involved in the raffinose oligosaccharide pathway, involving the synthesis of galactinol and raffinose. The increased transcript abundance of these genes (Supplemental Table S2) in the ovary tissue suggests increases in the levels of galactinol and raffinose that may act as scavengers of ROS accumulated under drought (Foyer and Shigeoka, 2011; Knaupp et al., 2011; Valluru and Van den Ende, 2011). The decrease in transcript abundance of known antioxidant genes (Supplemental Table S6), with a resultant diminishment of the capacity to contain ROS levels, may not have been adequately compensated by the scavenging action of raffinose, since embryo abortion still took place. Genes involved in galactinol and raffinose synthesis have W-box cis-elements that are bound by SnRK1. Together with bZIP11 and T6P, SnRK1 forms a regulatory network that operates under carbon-starvation conditions (Hanson and Smeekens, 2009; Valluru and Van den Ende, 2011). The data show that SnRK1 had increased transcript abundance (Table IV) in the drought-stressed ovary tissue, in agreement with the increased expression of raffinose oligosaccharide pathway-associated genes. However, bZIP11 showed decreased transcript abundance in drought-stressed ovary. The increased transcript abundance of several TPS-encoding genes (Supplemental Table S7) suggests an increase in T6P levels. The SnRKI, bZIP11, and T6P regulatory network works under low-sugar and -energy conditions by repressing carbon metabolism-associated genes involved in biosynthesis and up-regulating those genes involved in catabolism. The pattern observed for starch and Suc metabolism-associated genes in drought-stressed ovaries suggests such an influence of the SnRK1 complex. The responses reported here that are related to raffinose and trehalose metabolism-associated genes were also reported by Zhuang et al. (2007) for drought-stressed ear and tassels that were undergoing meiosis. This suggests that these stress responses, at least, are not specific to fertilized ovaries but also occur at earlier stages.

Cell Cycle and Cell Division Are Affected by Drought Stress

The genes in the GO categories "cell cycle" and "cell division" that showed decreased transcript abundance in the ovary tissue but were unaffected in the leaf meristem suggest that differentiation processes were affected. This includes ZmRBR2, which has been suggested to have a role in cell cycle events and endoreduplication in the maize endosperm (Grafi et al., 1996). Regulation of the cell cycle under healthy conditions is a carefully orchestrated web of interactions with many cellular participants and synchronized constraints. The S and M phases (DNA synthesis and cell mitosis) are largely constant in length, while the G1 and G2 phases are highly variable. Entering into S phase and the following M phase sets the cycle into an irreversible forward direction. If the cell is not capable of completing the cycle, due to insufficient cellular resources, the result can be cell death. Because of the severity of the outcome, cell division is highly regulated and coordinated (de Jager et al., 2005).

In response to drought stress in maize ovary, a host of genes encoding cell cycle factors promoting cell cycle progression showed decreased transcript abundance, and the few factors that inhibit cell cycle progression showed increased transcript abundance (Fig. 6). Under drought, ovary tissue appears to experience a drastic perturbation to the cell cycle, which will devastate cell proliferation capabilities. Progression through the cell cycle is driven by cyclin-dependent kinases with cyclins as regulatory subunits. Plant cyclins are typically categorized into four classes, A to D. A-type cyclins are present S phase to M phase and B-type cyclins are maximized at the G2-M transition and M phase. D-type cyclins regulate the G1-S transition (De Veylder et al., 2007). Genome wide, maize

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

possesses 59 cyclins spread over 10 chromosomes (Hu et al., 2010). In wheat, cyclin expression is reduced and cells accumulate in the G1 and G2 phases under drought stress. Early endosperm development is particularly vulnerable to drought stress, possibly because of the heavy mitotic requirements (Kitsios and Doonan, 2011). As shown in Figure 6, of 11 cyclin genes, all except for two showed decreased transcript abundance in drought-stressed ovary tissue (cyclinD4 showed increased transcript abundance and a cyclin δ was undetected). In leaf meristem, three cyclins were detected with increased expression.

The plant retinoblastoma-related (RBR) family contains one to three RBR genes. Maize is the only current example with three family members, dicots contain one, and in general, monocots contain two. ZmRBR1 and ZmRBR3 are involved in G1-S transition control through the adenovirus E2 promoter factor pathway. ZmRBR1 acts as an inhibitor of ZmRBR3. ZmRBR1 inhibits the cell cycle through effects on chromatin remodeling, and ZmRBR3 exerts a positive influence through controlling DNA replication and minichromosome maintenance (Sabelli and Larkins, 2009). The transition from mitotic cell cycle to the endoreduplication cell cycle (G/S phases producing polyploid DNA content) in endosperm is associated with low *ZmRBR3* and increased *ZmRBR1* gene expression (Sabelli et al., 2009). *ZmRBR2*, whose role is unknown, is constitutively expressed at 50% of *ZmRBR1* levels (Najera-Martinez et al., 2008). Our experimental results show a decrease in expression of *ZmRBR3* and nonsignificant increases of *ZmRBR1* and *ZmRBR2*. This drought-stressed expression pattern does not match healthy cell cycle or endoreduplication cycle expectations.

CDKIs promote G1 arrest in the cell cycle. In maize endosperm tissue, mutants overexpressing the CDKI, Zeama;KRP;1, counter retinoblastoma-related protein function, displaying endoreduplication without division (Coelho et al., 2005). Modulation of the cyclin-dependent kinases plays an important role in the transition from mitotic cell cycle to the endoreduplication cell cycle (Sabelli and Larkins, 2009). Two CDKIs (CDKI and CDKI1) showed increased transcript abundance in ovary tissue. A third CDKI, CDKI2, showed increased transcript abundance below the cutoff value. The increase in expression of the CDKIs is consistent with disrupted cell cycle progression in the drought-stressed ovary tissue.

In yeast and humans, RAD17 participates in a checkpoint protein complex involved in DNA damage G2 cell cycle arrest. In Arabidopsis, RAD17 homolog (*AtRAD17*) mutants show increased sensitivity to DNA-damaging chemicals and delay in double-strand break (DSB) repair. The frequency of interchromosomal homologous recombination increases along with the general deregulation of repair (Heitzeberg et al., 2004). An Arabidopsis *rad9-rad17* double mutant showed no induction of a ribonucleotide reductase subunit (*AtRNR2b*) and could not sustain nucleotide production for repair (Roa et al., 2009). Our data show that RAD17 expression was decreased in ovary tissue and unresponsive in leaf meristem.

In yeast, RAD51 plays a key role in DSB/DNA repair and homologous recombination. In maize, RAD51 has a similar role. Double mutants of the RAD51 homologs show male sterility, reduced seed set, and a high susceptibility to DSBs (Li et al., 2007). MAD2 was originally identified in budding yeast as a highly conserved protein involved in the spindle checkpoint system, ensuring that the metaphase is complete before the progression to anaphase. A similar role has been demonstrated for the maize MAD2 homolog, with the localization of the MAD2 homolog to the prometaphase kinetochore (Yu et al., 1999). RAD51 showed decreased transcript abundance in maize drought-stressed ovary tissue and was undetected in leaf meristem. The decreased expression of both RAD17 and RAD51 suggests a disruption of cell cycle control.

Nuf2, in yeast, is associated with the spindle pole body and a candidate for spindle pole body separation. Work with mutants demonstrated that Nuf2 is required for nuclear division. Using antibodies to yeast Nuf2, a similar protein was found in the spindle pole body of mammals (Osborne et al., 1994). There is no direct evidence for the role of a Nuf2 homolog in plants, except sequence homology. Nuf2 exhibited decreased expression in drought-stressed ovary tissue, contrary to the expected increased expression in a rapidly dividing tissue. Expression of Nuf2 in the leaf meristem was not detected.

Cell cycle progression also depends on environmental nutritional cues as a measure of carbon availability. Arabidopsis meristem growth-arrested *stip* mutants can be rescued from G2 arrest by providing an exogenous source of carbohydrates. Sugars activate key cell cycle regulators and influence the regulation of the G2-M transition (Skylar et al., 2011). The expression data suggest disrupted sugar metabolism under drought stress and likely an additional influence on cell cycle progression.

The magnitude and direction of expression changes among the host of cell cycle and cell division genes in both tissues indicate a significant disturbance of the cell cycle in ovary tissue and suggest a process leading to embryo abortion. An overview of all the above influences on the cell cycle is illustrated in Figure 6.

PCD Gene Responses

With the severe disrupting effects of drought stress on ovary tissue, the question arises about the role of PCD in the failing embryo and endosperm. PCD plays a crucial role in organism development and survival. In development, organized destruction of cells is important in organ and tissue formation. For survival, PCD provides a means of removing damaged cells in an orchestrated and safe way. Perturbed cells respond to the severity of stress by mitigating damage through

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

ROS scavenging when minor, PCD when major, and necrosis when overwhelming. PCD in plants is less studied than in animals and exhibits significant differences from animal PCD. Cells destroyed by plant PCD are not engulfed and remain suspended in the cell wall in both developmental and survival PCD.

After identifying several known PCD genes (Supplemental Table S5), the RNA-Seq data were mined for these genes or their homologs with significant expression changes under drought stress. Four categories of genes with significant expression changes were identified: lsd, metacaspases (including lol genes), mlo family, and bax inhibitors. lsd1 (for lesions simulating disease resistance response) is a negative regulator of cell death in the hypersensitive response initiated by localized superoxide production in Arabidopsis and suspected to reduce cell death spread (Dietrich et al., 1994). Drought-stressed ovary tissue shows a marked decrease in expression of the lsd1 homolog.

Metacaspases are Cys-dependent Arg/Lys-specific proteases found in plants, fungi, and protozoa playing a similar role to caspases in animal PCD, stress, and cell proliferation (Tsiatsiani et al., 2011). The regulation of caspases is typically posttranslational for rapid response. Maize ovaries show increased expression of two metacaspases (annotated as *lol3* and *metacaspase1*) and decreased transcript abundance of two metacaspases (annotated as *metacaspase5* and *metacaspase type II*). In contrast, leaf meristem tissue showed increased expression of only *metacaspase5*.

The Mlo (modulator of defense and death) family is plant specific and known to be associated with stress in several plants. This gene family was originally identified in barley, where the use of mutants demonstrated spontaneous PCD in the absence of inductive signals and the genes were inferred to be negative regulators of PCD (Piffanelli et al., 2002). Seven wheat Mlo proteins were found to be tissue specific and unresponsive to salt or osmotic stress in roots and shoots (Konishi et al., 2010). However, all five significantly responsive maize Mlo genes showed decreased transcript abundance in drought-stressed ovary tissue. Drought-stressed leaf meristem tissue showed increased expression of *Mlo4*.

BAX inhibitor I, an endoplasmic reticulum-resident transmembrane protein, is ubiquitous in eukaryotes as a cell death suppressor (Ishikawa et al., 2011). Of the two BAX inhibitor genes represented by transcripts, one showed increased transcript abundance in leaf meristem tissue and no significant change in ovary tissue. The other gene displayed decreased transcript abundance in ovary tissue, possibly contributing to increased PCD.

The negative regulators of PCD, the lsd and mlo family genes, showed decreased transcript abundance. Between the two BAX inhibitor genes, both negative regulators of PCD, one showed no significant change and the other showed decreased transcript abundance in ovary tissue. The positive regulators of PCD, the metacaspases, showed a mixed profile, with increased expression of metacaspase1 genes and decreased transcript abundance of metacaspase5 and type II. The expression profiles of these PCD-related genes support the conclusion of increased PCD occurring in drought-stressed ovary tissues.

ABA-Related Effects

ABA is a major plant hormone that plays a key role in adaptive responses to drought stress (Cutler et al., 2010). ABA regulates a large number of genes in Arabidopsis (Fujita et al., 2011). The first committed step in ABA synthesis is catalyzed by the NCED gene family (Schwartz et al., 1997; Dolferus et al., 2011). The extent of stress-mediated synthesis of ABA is also influenced by the zeaxanthin epoxidase gene family (Frey et al., 1999). A drought-mediated increase in ABA levels has been reported at the time of anthesis in maize exposed to stress for longer periods before pollination (Yu and Setter, 2003; Setter et al., 2011). In agreement with these findings, the data reported here show increased transcript abundance of ABA biosynthesis genes in the ovary tissue (Supplemental Table S7). Genes with weak similarity to the AAO class in Arabidopsis and known to catalyze the last step in ABA synthesis showed decreased transcript abundance in ovary tissue. However, *AAO3*, which is the gene specifically responsible for ABA synthesis in seeds (Seo et al., 2004), was unaffected by exposure to drought in either tissue. In fact, previous studies have shown that single loss-of-function mutants of *AAO1* and *AAO4* (Seo et al., 2004; decreased transcript abundance in this study) did not change endogenous ABA levels when compared with the wild type.

A large number of transcription factors are known to be involved in ABA-mediated responses. Major positive regulators of ABA-mediated responses include the transcription factors belonging to the bZIP class (Fujita et al., 2011). Members of the bZIP gene family are known to regulate important cellular processes in all eukaryotes (Jakoby et al., 2002; Corrêa et al., 2008; Fujita et al., 2011). An important transcription factor in this class is ABI5, a positive regulator of ABA signaling, which is expressed in seeds and mainly serves as a developmental checkpoint (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Bensmihen et al., 2005). The increased expression of the genes involved in ABA synthesis and the ABI5 transcription factor in the drought-stressed ovary tissue suggests the activation of the ABA-mediated response pathway that induces the expression of many genes that play an important role in drought responses (Finkelstein et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Members of the ABF gene family are known to be induced in vegetative tissue under drought stress (Choi et al., 2000; Kang et al., 2002; Fujita et al., 2005, 2011), which is in accordance with the increased transcript abundance of the ABF4 gene in the leaf meristem

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

observed in this report (Supplemental Table S7). The late embryogenesis abundant proteins that showed increased transcript abundance in the ovary tissue belong to another of the many classes of ABA-induced genes.

Responses of Antioxidant Genes

Many genes in the enriched GO category "response to oxidative stress" (Supplemental Table S6) showed decreased expression by drought in ovaries, whereas the reverse, and expected, response was observed in leaf meristem, suggesting that antioxidant defense processes were failing in the ovary under the level of drought stress imposed (e.g. several ascorbate peroxidase genes and copper/zinc- and manganese-superoxide dismutases responded negatively to the stress). Thus, scavenging of ROS through the action of antioxidant genes encoding enzymes that reside in the mitochondrion and the cytosol appears to be curtailed under drought stress in the ovary, whereas the drought stress did not have this effect on antioxidant gene expression in leaf meristem, where antioxidant defenses occurred, as has been observed many times, in many tissues, and in many species. An analogous decrease in the expression of antioxidant genes was observed in a more drought-susceptible accession of Andean potato when compared with patterns of gene expression in a more drought-tolerant accession (Mane et al., 2007, 2008). Interestingly, catalase and peroxisomal APX showed increased expression in the stressed ovary, which is the more expected response to drought (Supplemental Table S3). Any antioxidant resistance response that occurred in the peroxisome may have been insufficient to stem the tide of ROS arising under drought conditions. The decreased expression of an APX1 homolog in the drought-stressed maize ovaries also suggests a suppression of the well-established resistance signaling pathway that essentially mounts a resistance to oxidative stress, including the protection of chromosomal DNA that arises in response to drought, among many other abiotic stresses (Vanderauwera et al., 2011; Suzuki et al., 2012).

Phospholipase- and PI Signaling-Associated Genes

PLC catalyzes the conversion of membrane-bound PI species into phosphorylated inositol isoforms and diacylglycerol, each of which participates in PI signaling. The variety of isoforms and phosphorylation possibilities provides a signal molecule with subtle complexity and context. PLC transcript levels have been shown to vary under stress conditions (Gillaspy, 2011). All PLC genes in the maize drought data set showed decreased expression or insignificant changes in ovary under drought stress, while an extreme decrease in transcript abundance of PI-specific phospholipase C4 was observed in leaf meristem tissue (Supplemental Table S8). This decreased transcript abundance is likely to have a significant effect on the availability of inositol 1,4,5-triphosphate [Ins(1,4,5)P3] for signal transduction in both tissues. That being the case, it is unlikely that Ins(1,4,5)P_3-associated signaling plays a role in the differential drought sensitivity of ovary tissue, compared with the vegetative parts of the maize plant. Other genes associated with Ins(1,4,5)P_3 signaling, 5PTase and PIP5K, show mixed patterns of changes in both tissues, on the other hand, yielding inconclusive results.

PLDs are involved in plant growth, development, and environmental responses (e.g. stomatal movement, hyperosmotic, ROS, and pathogen responses; Bargmann and Munnik, 2006; Xue et al., 2009; Hong et al., 2010). Enzymatic activity of members of the PLD gene family results in the production of phosphatidic acid, another major signaling molecule that responds to abiotic stress (Li et al., 2009). In the case of drought stress, increased PLDα1 activity results in the activation of ABA signaling (Hong et al., 2010). Arabidopsis plants down-regulated with respect to PLDα expression were found to be more drought sensitive than their corresponding wild-type controls (Mane et al., 2007). However, in the case of the PLDα1 gene expression results reported here, the data are difficult to interpret. Five significantly responding PLDα genes showed mixed changes in the drought-stressed ovary tissue (Supplemental Table S8). In contrast, one gene encoding a PLDα1 homolog (GRMZM2G019029) responded positively in both leaf meristem and ovary tissue.

In contrast, PLDδ, which is known to be involved in ROS responses and cell viability in Arabidopsis, showed decreased expression in drought-stressed maize ovary tissue but not in the leaf meristem. This result is in agreement with the finding that cell cycle genes and major ROS-scavenging genes showed decreased expression in drought-stressed ovary tissue but not in the leaf meristem, while PCD-associated genes showed increased transcript abundance. Taken together, these data suggest that the signaling pathway in which PLDδ participates was specifically down-regulated in the ovary tissue under drought stress. Repression of this defense pathway is correlated with a possible failure of antioxidant processes and of signaling mediated through regulatory genes such as APX1, leading to arrest of the cell cycle and eventual cell death.

Working Hypothesis

The data suggest that what appear to be crucial stress signaling events are impaired in the drought-stressed maize ovaries, whereas the same suppression did not occur in leaf meristem exposed to the same stress dosage at the whole plant level. Taken together, ABA levels appear to have increased in the drought-stressed ovaries but increased to a lesser extent in the leaf meristem. The resultant ABA action may have

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

caused the observed repression of both cell wall and soluble invertase expression in the stressed ovary tissues. The repression of the soluble invertase compounded the effect of lowered cell wall invertase with respect to its metabolic function, since less substrate was available to associate with mitochondrial hexokinase to provide ADP to maintain coupled electron transport (Xiang et al., 2011). Hexokinase, as a signaling molecule, sensed a decrease in available Glc and activated the SnRK1 pathway. However, this same hexokinase was less functional metabolically, because less Glc was available. Under these conditions, increased ROS arose in the mitochondrion due to a lack of substrate, to the point where genes encoding components of ROS-scavenging pathways in the mitochondrion and the cytosol were repressed, a symptom of high oxidative stress, correlated with greater stress susceptibility. The increases in catalase and of APX associated with the peroxisome appeared not to have been sufficient to detoxify ROS accumulating in the stressed ovary. ABA action, and disruption of crucial hexose-Suc ratios, due to the repression of invertases, resulted in a process comparable to the ABA-mediated acceleration of senescence, shown in leaves by others (Ruan et al., 2010). This may be related to the action of SnRK1, since DIN1, a SEN gene, and SnRK1 target genes showed increased expression in the stressed ovaries. SnRK1 also may have inhibited AGPase expression, and a coregulation mechanism led to decreases in the expression of other genes related to starch biosynthesis.

Inferences and hypotheses proposed in this report with respect to drought-mediated events in the ovary

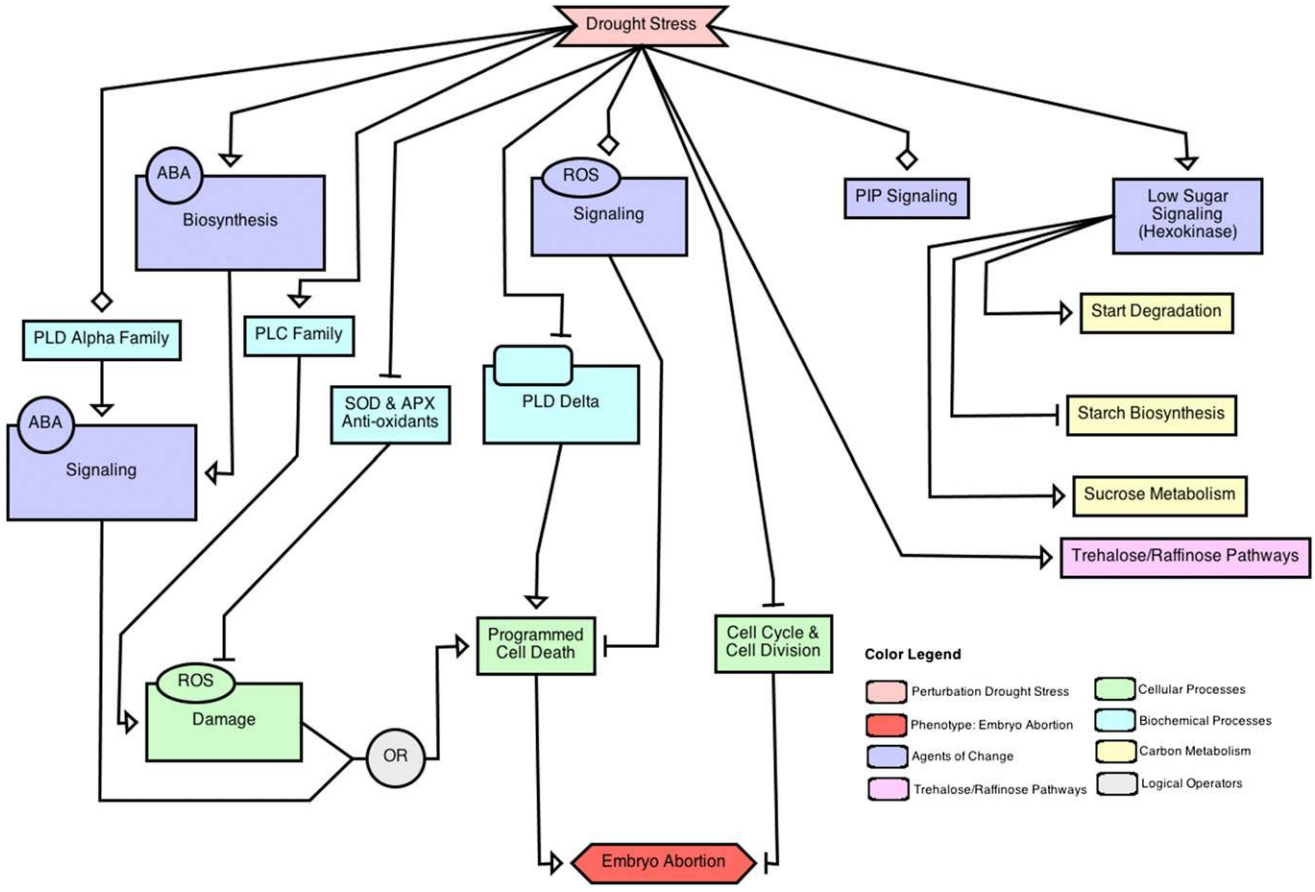


Figure 8. Overview of drought stress effects on maize ovary tissue at 1 DAP. The core figure is drawn according to Systems Biology Graphical Notation Activity Flow specifications (Level 1 Version 1) using Virginia Tech's Beacon software. Shaded colors group glyphs with similar processes or events. The relationships, or arcs, express the nature of the influence (positive, negative, or unknown) between the glyphs and summarize this paper's findings along with support from the literature. References Zinselmeier et al. (1999), Andersen et al. (2002), and Ruan et al. (2010) support and provide metabolite measurements for the carbon metabolism relationships under drought perturbation. Reference Zhuang et al. (2007) supports the positive influence of drought on the trehalose/raffinose pathway. References Hanson and Smeekens (2009) and Bolouri-Moghaddam et al. (2010) substantiate the positive influence on low-sugar signaling. Reference Hong et al. (2010) and the review by Li et al. (2009) associate PLDα and PLDδ expression to ROS and drought stress. References Yu and Setter (2003) and Setter et al. (2011) document increases in ABA under drought stress.

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

tissue are presented in Figure 8. The signature of gene expression observed in the drought-stressed maize ovaries at 1 DAP is in agreement with the activation of PCD, the halting of the cell cycle, impaired drought, and heightened carbon starvation signaling. These events may have been mediated by ABA, cell wall and soluble invertases, and increases in ROS to toxic levels due to mitochondrial malfunctioning and perhaps also in other subcellular locations. The data suggest that these events did not take place in the leaf meristem, where antioxidant defense mechanisms operated successfully and the downstream changes associated with cell death and PCD did not occur. Interestingly, the gene expression data suggest that PI signaling may be impaired in both tissues, since PLC mRNA levels were decreased by drought in both data sets.

MATERIALS AND METHODS

Plant Material and Drought Stress Conditions

Maize (*Zea mays*) plants of inbred line B73 were grown in 10-L pots with a 1:1:1 mix of peat:vermiculite:perlite with 6 g of pulverized limestone, 35 g $CaSO_4$, 42 g of powdered $FeSO_4$, and 1 g of trace fritted element (Setter and Flannigan, 2001). The plants were hand irrigated daily to maintain soil water content close to field capacity, and the nutrients were supplied on a weekly basis with a general purpose fertilizer (15-16-17; Scott-Sierra Horticultural Products). Plants were grown under well-watered conditions until they reached the reproductive stage (at the onset of silk emergence), when irrigation was withheld for one-half of the plants. Two to 3 d after irrigation was withheld, the plants were hand pollinated, and 24 h after pollination, measurements and samples were collected for transcriptome analysis. At this stage, drought-stressed plants had undergone 3 or 4 d of drought stress, while controls were well watered throughout this period. At the end of the drought period (1 DAP), the basal leaf meristem of the three youngest leaves and the ovary tissues (Andersen et al., 2002; Boyer and McLaughlin, 2007) were sampled for Illumina deep sequencing. Samples were labeled as well-watered control leaf meristem, well-watered control ovaries/"cob," drought-stressed leaf meristem, and drought-stressed ovary tissue.

Total RNA, Poly(A) RNA Isolation, and Library Preparation

Two biological replicates were used for all RNA-Seq experiments from each tissue type. The total RNA from the leaf meristem and ovary tissues was extracted (1 DAP) using Trizol reagent (Invitrogen) and purified using the RNeasy Plant Mini kit (Qiagen). On-column DNase digestion was performed according to the manufacturer's protocol (Qiagen). The integrity and quality of the total RNA was checked by a NanoDrop 1000 spectrophotometer and formaldehyde-agarose gel electrophoresis. The poly(A) RNA was isolated from purified total RNA using poly(T) oligonucleotide-attached magnetic beads. Following purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature, and the cleaved RNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers. Second-strand cDNA synthesis was done using DNA Polymerase I and RNaseH, and the cDNA fragments were processed for end repair, an addition of a single "A" base, and ligation of the adapters. These products were then purified and enriched by PCR to create the final cDNA library and sequenced on the Illumina Genome Analyzer IIx system according to the manufacturer's recommendations (Illumina).

Processing and Mapping of Illumina Reads

The RNA-Seq reads generated by the Illumina Genome Analyzer were initially processed to remove the adapter sequences and low-quality bases at the 3′ end. This resulted in reads with lengths ranging from as low as 12 bp to as high as 76 bp. Reads greater than 24 bp were considered for further analysis, and the cumulative read length distribution for the eight libraries is shown in Supplemental Figure S3.

After preprocessing the RNA-Seq data, the reads were mapped to the maize masked genome using a spliced aligner called Tophat (Trapnell et al., 2009; release 5b, downloaded from http://ftp.maizesequence.org/current/) that allows the identification of splicing events involving novel exons and novel intergenic transcripts. Tophat first maps reads using an unspliced aligner, and the remaining unmapped reads are split into shorter segments and aligned independently. Based on the alignment of these shorter segments, the genomic regions are joined and searched for spliced alignments. Default Tophat parameters, which allow up to two mismatches and report up to 40 alignments for reads mapping at multiple positions, were used.

Transcript Assembly Using Cufflinks

The Sequence Alignment/Map files generated by Tophat were provided as input to the software Cufflinks (Trapnell et al., 2010), which assembles the alignments in the Sequence Alignment/Map file into transfrags. Cufflinks does this assembly independently of the existing gene annotations. Cufflinks constructs a minimum set of transcripts that best describes the RNA-Seq reads. The unit of measurement used by Cufflinks to estimate transcript abundance is FPKM. The Cufflinks statistical model probabilistically assigns reads to the assembled isoforms.

Comparison with Reference Annotation Using Cuffmerge

The reproductive tissue (ovaries) and the vegetative tissue (basal leaf meristem) each have four libraries corresponding to well-watered and drought-stressed tissue with one biological replicate each. Thus, four assemblies/tissue types were generated by Cufflinks in each tissue. Cuffmerge (Trapnell et al., 2010) was used to merge these four assemblies with the reference annotation (ZmB73_5b_FGS.gff) downloaded from http://ftp.maizesequence.org/current/ into a single gtf file that was used later to identify differentially expressed genes. The class codes in the Cuffmerge output were used to identify novel isoforms, intergenic transcripts, and splice junctions.

Differential Expression

The logarithm of the FPKM (Supplemental Fig. S1) values is normally distributed and homoscedastic in nature (homogeneity of variance). Limma (Smyth, 2004) was used, which exploits these properties of log FPKM values to make the analysis more robust for small numbers of samples by fitting a linear model to the expression data for each gene by borrowing information across genes using an empirical Bayesian method. The Limma R package was downloaded from http://bioconductor.org/packages/release/bioc/html/limma.html. The lmFit function was used to estimate fold change and SE by fitting a linear model for each gene. The eBayes function was used to apply empirical Bayes smoothing of the SE values. Limma outputs the *T* statistic and the *P* values for each gene. The QVALUE R package (Storey, 2002), downloaded from http://genomics.princeton.edu/storeylab/qvalue/, was used to calculate the *q* values from the *P* values obtained from Limma. A very stringent cutoff, 0.05 *q* value and fold change value of 2, was used for identifying differentially expressed genes.

GO Enrichment

The GO functional annotations for maize gene products were downloaded from the AgBase Web site (http://www.agbase.msstate.edu/cgi-bin/information/Maize.pl). Only the "biological process" category was considered for the functional enrichment analysis. In the maize annotation used, approximately 8,830 genes were annotated to GO biological process terms (as of November 2011). For GO enrichment analysis, the algorithm of Kim and Volsky (2005), also known as parametric analysis of gene set enrichment, was implemented. The input for this algorithm is a list of genes and a parameter that can be either a test-statistic or log-fold change; here, the *T* statistic was used as the parameter. The result of the enrichment analysis is a list of GO terms and their corresponding Z scores. The Z score for each term is given by the following formula: $Z = (Sm - \mu) \times m^{1/2}/\delta$, where *Sm* is the mean *T* statistic value for genes annotated to that GO term, μ is the mean *T* statistic value of the entire gene list, and δ is the SD of the *T* statistic value in the entire gene list.

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

P values were calculated for each GO term from their Z score and were corrected for multiple hypothesis testing using the QVALUE R package (Storey, 2002). A *q* value cutoff of 0.05 was used to select enriched GO terms.

Use of MapMan to Identify Drought-Responsive Pathways

The MapMan tool facilitates the classification of transcripts (as well as other biological entities) into hierarchical categories (known as bins) in a manner that alleviates the redundancy present in other commonly used ontologies (Usadel et al., 2009); therefore, the tool provides an additional level of analysis beyond the functional enrichment of typical GO categories. Using this schema, the user may view a metabolic pathway or process of interest annotated by groups of participatory entities (maize transcripts, in this case), where each entity within a given group is represented by a discrete signal visualized using intensity of color (Thimm et al., 2004). Additionally, the tool includes a variety of relevant statistical packages (Usadel et al., 2005) and the ability to visually filter data based on user-defined statistical cutoffs.

qRT-PCR Analysis

Real-time PCR was carried out using a Bio-Rad iQ5 thermo cycler. The comparative C_T method of quantitation was used with maize β-tubulin and Actin1 as references and was amplified in parallel with the target gene(s), allowing gene expression normalization and providing quantification. The relative fold change for each of the selected genes was determined for both the control and drought-stressed plants. Two independent biological replicates of each sample and three technical replicates of each biological replicate were used for real-time PCR analysis. For each sample, 1 μg of total RNA from one of the biological replicates was converted into cDNA using oligo(dT) 15-mer and SuperScript III reverse transcriptase (Invitrogen Life Technologies). This cDNA was diluted to 250 μL in sterile water. Validation experiments were performed on five to six log dilutions of each of the target genes together with the reference genes to determine if the amplification efficiencies were equal. qRT-PCR was performed using iQSYBR Green Supermix (Bio-Rad). Melting-curve analysis, by applying increasing temperature from 55°C to 95°C (0.5°C per 10 s), and gel electrophoresis of the final product confirmed single amplicons. Negative control reactions using untranscribed RNA were also run to confirm the absence of genomic DNA. To determine relative fold differences for each sample in each experiment, the C_T value for each gene was normalized to the C_T value for the reference gene and was calculated relative to a calibrator using the $\Delta\Delta C_T$ method as described (Livak and Schmittgen, 2001).

Sequence data from this article can be found in the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE40070.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Box plot of the log FPKM expression values.

Supplemental Figure S2. Box-plot distribution of coefficient of variation, in percentage, among biological replicates.

Supplemental Figure S3. Illumina read-length distribution in the maize ovary and leaf meristem libraries.

Supplemental Table S1. Genes with FPKM, an expression measure, greater than 5 in select GO terms related to growth and development.

Supplemental Table S2. Genes annotated to raffinose and galactinol synthases.

Supplemental Table S3. Genes annotated to TPS and trehalose phosphate phosphatase.

Supplemental Table S4. Maize drought stress gene expression for cell cycle-related genes.

Supplemental Table S5. Maize gene expression for PCD-related genes in drought-stressed ovary and leaf meristem tissues.

Supplemental Table S6. Genes annotated to the GO term "response to oxidative stress."

Supplemental Table S7. Maize genes involved in ABA synthesis and ABA-mediated response as classified by MapMan.

Supplemental Table S8. Genes annotated to phospholipase in the data set.

Supplemental Table S9. Primer sequences used to amplify the invertase class of genes in maize ovary and leaf meristem tissues.

ACKNOWLEDGMENTS

We are grateful to Drs. Glenda Gillaspy (Virginia Tech), Lenwood Heath (Virginia Tech), T.M. Murali (Virginia Tech), and Eva Collakova (Virginia Tech) for expert advice afforded to us during the course of our analysis of the data and the preparation of the manuscript.

Received May 15, 2012; accepted July 19, 2012; published July 26, 2012.

LITERATURE CITED

Andersen MN, Asch F, Wu Y, Jensen 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

Arroyo A, Bossi F, Finkelstein RR, León P (2003) Three genes that affect sugar sensing (abscisic acid insensitive 4, abscisic acid insensitive 5, and constitutive triple response 1) are differentially regulated by glucose in Arabidopsis. Plant Physiol **133:** 231–242

Baena-González E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signalling. Nature **448:** 938–942

Bargmann BO, Munnik T (2006) The role of phospholipase D in plant stress responses. Curr Opin Plant Biol **9:** 515–522

Bate NJ, Niu X, Wang Y, Reimann KS, Helentjaris TG (2004) An invertase inhibitor from maize localizes to the embryo surrounding region during early kernel development. Plant Physiol **134:** 246–254

Bensmihen S, Giraudat J, Parcy F (2005) Characterization of three homologous basic leucine zipper transcription factors (bZIP) of the ABI5 family during Arabidopsis thaliana embryo maturation. J Exp Bot **56:** 597–603

Bolouri-Moghaddam MR, Le Roy K, Xiang L, Rolland F, Van den Ende W (2010) Sugar signalling and antioxidant network connections in plant cells. FEBS J **277:** 2022–2037

Boyer JS, McLaughlin JE (2007) Functional reversion to identify controlling genes in multigenic responses: analysis of floral abortion. J Exp Bot **58:** 267–277

Camacho-Pereira J, Meyer LE, Machado LB, Oliveira MF, Galina A (2009) Reactive oxygen species production by potato tuber mitochondria is modulated by mitochondrially bound hexokinase activity. Plant Physiol **149:** 1099–1110

Cao H, James MG, Myers AM (2000) Purification and characterization of soluble starch synthases from maize endosperm. Arch Biochem Biophys **373:** 135–146

Chen J, Huang B, Li Y, Du H, Gu Y, Liu H, Zhang J, Huang Y (2011) Synergistic influence of sucrose and abscisic acid on the genes involved in starch synthesis in maize endosperm. Carbohydr Res **346:** 1684–1691

Cho YH, Sheen J, Yoo SD (2010) Low glucose uncouples hexokinase1-dependent sugar signaling from stress and defense hormone abscisic acid and C2H4 responses in Arabidopsis. Plant Physiol **152:** 1180–1182

Cho YH, Yoo SD, Sheen J (2006) Regulatory functions of nuclear hexokinase1 complex in glucose signaling. Cell **127:** 579–589

Choi H, Hong J, Ha J, Kang J, Kim SY (2000) ABFs, a family of ABA-responsive element binding factors. J Biol Chem **275:** 1723–1730

Chourey PS, Jain M, Li QB, Carlson SJ (2006) Genetic control of cell wall invertases in developing endosperm of maize. Planta **223:** 159–167

Coelho CM, Dante RA, Sabelli PA, Sun Y, Dilkes BP, Gordon-Kamm WJ, Larkins BA (2005) Cyclin-dependent kinase inhibitors in maize endosperm and their potential role in endoreduplication. Plant Physiol **138:** 2323–2336

Corrêa LG, Riaño-Pachón DM, Schrago CG, dos Santos RV, Mueller-Roeber B, Vincentz M (2008) The role of bZIP transcription factors in green plant evolution: adaptive features emerging from four founder genes. PLoS ONE **3:** e2944

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

Couée I, Sulmon C, Gouesbet G, El Amrani A (2006) Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. J Exp Bot **57:** 449–459

Cruz de Carvalho MH (2008) Drought stress and reactive oxygen species: production, scavenging and signaling. Plant Signal Behav **3:** 156–165

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol **61:** 651–679

de Jager SM, Maughan S, Dewitte W, Scofield S, Murray JA (2005) The developmental context of cell-cycle control in plants. Semin Cell Dev Biol **16:** 385–396

De Veylder L, Beeckman T, Inzé D (2007) The ins and outs of the plant cell cycle. Nat Rev Mol Cell Biol **8:** 655–665

Dietrich RA, Delaney TP, Uknes SJ, Ward ER, Ryals JA, Dangl JL (1994) Arabidopsis mutants simulating disease resistance response. Cell **77:** 565–577

Dolferus R, Ji X, Richards RA (2011) Abiotic stress and control of grain number in cereals. Plant Sci **181:** 331–341

Feng HY, Wang ZM, Kong FN, Zhang MJ, Zhou SL (2011) Roles of carbohydrate supply and ethylene, polyamines in maize kernel set. J Integr Plant Biol **53:** 388–398

Finkelstein RR, Gampala SS, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell (Suppl) **14:** S15–S45

Finkelstein RR, Lynch TJ (2000) The *Arabidopsis* abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor. Plant Cell **12:** 599–609

Foyer CH, Shigeoka S (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol **155:** 93–100

Frey A, Audran C, Marin E, Sotta B, Marion-Poll A (1999) Engineering seed dormancy by the modification of zeaxanthin epoxidase gene expression. Plant Mol Biol **39:** 1267–1274

Fujita N, Yoshida M, Kondo T, Saito K, Utsumi Y, Tokunaga T, Nishi A, Satoh H, Park JH, Jane JL, et al (2007) Characterization of SSIIIa-deficient mutants of rice: the function of SSIIIa and pleiotropic effects by SSIIIa deficiency in the rice endosperm. Plant Physiol **144:** 2009–2023

Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. Plant Cell **17:** 3470–3488

Fujita Y, Fujita M, Shinozaki K, Yamaguchi-Shinozaki K (2011) ABA-mediated transcriptional regulation in response to osmotic stress in plants. J Plant Res **124:** 509–525

Gao M, Wanat J, Stinard PS, James MG, Myers AM (1998) Characterization of *dull1*, a maize gene coding for a novel starch synthase. Plant Cell **10:** 399–412

Gillaspy GE (2011) The cellular language of myo-inositol signaling. New Phytol **192:** 823–839

Grafi G, Burnett RJ, Helentjaris T, Larkins BA, DeCaprio JA, Sellers WR, Kaelin WG Jr (1996) A maize cDNA encoding a member of the retinoblastoma protein family: involvement in endoreduplication. Proc Natl Acad Sci USA **93:** 8962–8967

Hanson J, Smeekens S (2009) Sugar perception and signaling: an update. Curr Opin Plant Biol **12:** 562–567

Heitzeberg F, Chen IP, Hartung F, Orel N, Angelis KJ, Puchta H (2004) The Rad17 homologue of Arabidopsis is involved in the regulation of DNA damage repair and homologous recombination. Plant J **38:** 954–968

Hong Y, Zhang W, Wang X (2010) Phospholipase D and phosphatidic acid signalling in plant response to drought and salinity. Plant Cell Environ **33:** 627–635

Hu X, Cheng X, Jiang H, Zhu S, Cheng B, Xiang Y (2010) Genome-wide analysis of cyclins in maize (Zea mays). Genet Mol Res **9:** 1490–1503

Ishikawa T, Watanabe N, Nagano M, Kawai-Yamada M, Lam E (2011) Bax inhibitor-1: a highly conserved endoplasmic reticulum-resident cell death suppressor. Cell Death Differ **18:** 1271–1278

Jain M, Khurana JP (2009) Transcript profiling reveals diverse roles of auxin-responsive genes during reproductive development and abiotic stress in rice. FEBS J **276:** 3148–3162

Jakoby M, Weisshaar B, Dröge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F bZIP Research Group (2002) bZIP transcription factors in Arabidopsis. Trends Plant Sci **7:** 106–111

Ji X, Dong B, Shiran B, Talbot MJ, Edlington JE, Hughes T, White RG, Gubler F, Dolferus R (2011) Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. Plant Physiol **156:** 647–662

Jiang SY, Ramamoorthy R, Ramachandran S (2008) Comparative transcriptional profiling and evolutionary analysis of the GRAM domain family in eukaryotes. Dev Biol **314:** 418–432

Kang JY, Choi HI, Im MY, Kim SY (2002) *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14:** 343–357

Kaplan F, Guy CL (2004) β-Amylase induction and the protective role of maltose during temperature shock. Plant Physiol **135:** 1674–1684

Kim SY, Volsky DJ (2005) PAGE: parametric analysis of gene set enrichment. BMC Bioinformatics **6:** 144

Kitsios G, Doonan JH (2011) Cyclin dependent protein kinases and stress responses in plants. Plant Signal Behav **6:** 204–209

Knaupp M, Mishra KB, Nedbal L, Heyer AG (2011) Evidence for a role of raffinose in stabilizing photosystem II during freeze-thaw cycles. Planta **234:** 477–486

Konishi S, Sasakuma T, Sasanuma T (2010) Identification of novel Mlo family members in wheat and their genetic characterization. Genes Genet Syst **85:** 167–175

Kopka J, Provart NJ, Müller-Röber B (1997) Potato guard cells respond to drying soil by a complex change in the expression of genes related to carbon metabolism and turgor regulation. Plant J **11:** 871–882

Li J, Harper LC, Golubovskaya I, Wang CR, Weber D, Meeley RB, McElver J, Bowen B, Cande WZ, Schnable PS (2007) Functional analysis of maize RAD51 in meiosis and double-strand break repair. Genetics **176:** 1469–1482

Li M, Hong Y, Wang X (2009) Phospholipase D- and phosphatidic acid-mediated signaling in plants. Biochim Biophys Acta **1791:** 927–935

Li P, Ponnala L, Gandotra N, Wang L, Si Y, Tausta SL, Kebrom TH, Provart N, Patel R, Myers CR, et al (2010) The developmental dynamics of the maize leaf transcriptome. Nat Genet **42:** 1060–1067

Li Y, Lee KK, Walsh S, Smith C, Hadingham S, Sorefan K, Cawley G, Bevan MW (2006) Establishing glucose- and ABA-regulated transcription networks in Arabidopsis by microarray analysis and promoter classification using a Relevance Vector Machine. Genome Res **16:** 414–427

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **25:** 402–408

Lopez-Molina L, Chua NH (2000) A null mutation in a bZIP factor confers ABA-insensitivity in Arabidopsis thaliana. Plant Cell Physiol **41:** 541–547

Lou Y, Gou JY, Xue HW (2007) PIP5K9, an *Arabidopsis* phosphatidylinositol monophosphate kinase, interacts with a cytosolic invertase to negatively regulate sugar-mediated root growth. Plant Cell **19:** 163–181

Mane SP, Robinet CV, Ulanov A, Schafleitner R, Tincopa L, Gaudin A, Nomberto G, Alvarado C, Solis C, Bolivar LA, et al (2008) Molecular and physiological adaptation to prolonged drought stress in the leaves of two Andean potato genotypes. Funct Plant Biol **35:** 669–688

Mane SP, Vasquez-Robinet C, Sioson AA, Heath LS, Grene R (2007) Early PLDalpha-mediated events in response to progressive drought stress in Arabidopsis: a transcriptome analysis. J Exp Bot **58:** 241–252

Matiolli CC, Tomaz JP, Duarte GT, Prado FM, Del Bem LE, Silveira AB, Gauer L, Corrêa LG, Drumond RD, Viana AJ, et al (2011) The Arabidopsis bZIP gene AtbZIP63 is a sensitive integrator of transient abscisic acid and glucose signals. Plant Physiol **157:** 692–705

Najera-Martinez M, Ramirez-Parra E, Vazquez-Ramos J, Gutierrez C, Plasencia J (2008) Cloning and molecular characterisation of the maize retinoblastoma gene (ZmRBR2). Plant Sci **175:** 685–693

Ober ES, Setter TL, Madison JT, Thompson JF, Shapiro PS (1991) Influence of water deficit on maize endosperm development: enzyme activities and RNA transcripts of starch and zein synthesis, abscisic acid, and cell division. Plant Physiol **97:** 154–164

Oliver SN, Dennis ES, Dolferus R (2007) ABA regulates apoplastic sugar transport and is a potential signal for cold-induced pollen sterility in rice. Plant Cell Physiol **48:** 1319–1330

Osborne MA, Schlenstedt G, Jinks T, Silver PA (1994) Nuf2, a spindle pole body-associated protein required for nuclear division in yeast. J Cell Biol **125:** 853–866

Piffanelli P, Zhou F, Casais C, Orme J, Jarosch B, Schaffrath U, Collins NC, Panstruga R, Schulze-Lefert P (2002) The barley MLO modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. Plant Physiol **129:** 1076–1085

Poroyko V, Spollen WG, Hejlek LG, Hernandez AG, LeNoble ME, Davis G, Nguyen HT, Springer GK, Sharp RE, Bohnert HJ (2007) Comparing

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022

regional transcript profiles from maize primary roots under well-watered and low water potential conditions. J Exp Bot **58:** 279–289

Roa H, Lang J, Culligan KM, Keller M, Holec S, Cognat V, Montané MH, Houlné G, Chabouté ME (2009) Ribonucleotide reductase regulation in response to genotoxic stress in Arabidopsis. Plant Physiol **151:** 461–471

Ruan YL, Jin Y, Yang YJ, Li GJ, Boyer JS (2010) Sugar input, metabolism, and signaling mediated by invertase: roles in development, yield potential, and response to drought and heat. Mol Plant **3:** 942–955

Sabelli PA, Hoerster G, Lizarraga LE, Brown SW, Gordon-Kamm WJ, Larkins BA (2009) Positive regulation of minichromosome maintenance gene expression, DNA replication, and cell transformation by a plant retinoblastoma gene. Proc Natl Acad Sci USA **106:** 4042–4047

Sabelli PA, Larkins BA (2009) The contribution of cell cycle regulation to endosperm development. Sex Plant Reprod **22:** 207–219

Schwartz SH, Tan BC, Gage DA, Zeevaart JA, McCarty DR (1997) Specific oxidative cleavage of carotenoids by VP14 of maize. Science **276:** 1872–1874

Seo M, Aoki H, Koiwai H, Kamiya Y, Nambara E, Koshiba T (2004) Comparative studies on the Arabidopsis aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. Plant Cell Physiol **45:** 1694–1703

Setter TL, Flannigan BA (2001) Water deficit inhibits cell division and expression of transcripts involved in cell proliferation and endoreduplication in maize endosperm. J Exp Bot **52:** 1401–1408

Setter TL, Parra R (2010) Relationship of carbohydrate and abscisic acid levels to kernel set in maize under postpollination water deficit. Crop Sci **50:** 980–988

Setter TL, Yan J, Warburton M, Ribaut JM, Xu Y, Sawkins M, Buckler ES, Zhang Z, Gore MA (2011) Genetic association mapping identifies single nucleotide polymorphisms in genes that affect abscisic acid levels in maize floral tissues during drought. J Exp Bot **62:** 701–716

Skylar A, Sung F, Hong F, Chory J, Wu X (2011) Metabolic sugar signal promotes Arabidopsis meristematic proliferation via G2. Dev Biol **351:** 82–89

Smyth GK (2004) Linear Models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol **3:** 3

Staiger D, Green R (2011) RNA-based regulation in the plant circadian clock. Trends Plant Sci **16:** 517–523

Storey JD (2002) A direct approach to false discovery rates. J R Stat Soc Ser B Stat Methodol **64:** 479–498

Suzuki N, Koussevitzky S, Mittler R, Miller G (2012) ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ **35:** 259–270

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

Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, Selbig J, Müller LA, Rhee SY, Stitt M (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J **37:** 914–939

Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics **25:** 1105–1111

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol **28:** 511–515

Tsiatsiani L, Van Breusegem F, Gallois P, Zavialov A, Lam E, Bozhkov PV (2011) Metacaspases. Cell Death Differ **18:** 1279–1288

Usadel B, Nagel A, Thimm O, Redestig H, Blaesing OE, Palacios-Rojas N, Selbig J, Hannemann J, Piques MC, Steinhauser D, et al (2005) Extension of the visualization tool MapMan to allow statistical analysis of arrays, display of corresponding genes, and comparison with known responses. Plant Physiol **138:** 1195–1204

Usadel B, Poree F, Nagel A, Lohse M, Czedik-Eysenberg A, Stitt M (2009) A guide to using MapMan to visualize and compare omics data in plants: a case study in the crop species, maize. Plant Cell Environ **32:** 1211–1229

Valluru R, Van den Ende W (2011) Myo-inositol and beyond: emerging networks under stress. Plant Sci **181:** 387–400

Vanderauwera S, Suzuki N, Miller G, van de Cotte B, Morsa S, Ravanat JL, Hegie A, Triantaphylidès C, Shulaev V, Van Montagu MC, et al (2011) Extranuclear protection of chromosomal DNA from oxidative stress. Proc Natl Acad Sci USA **108:** 1711–1716

Vilhar B, Kladnik A, Blejec A, Chourey PS, Dermastia M (2002) Cytometrical evidence that the loss of seed weight in the *miniature1* seed mutant of maize is associated with reduced mitotic activity in the developing endosperm. Plant Physiol **129:** 23–30

Weigelt K, Küster H, Rutten T, Fait A, Fernie AR, Miersch O, Wasternack C, Emery RJ, Desel C, Hosein F, et al (2009) ADP-glucose pyrophosphorylase-deficient pea embryos reveal specific transcriptional and metabolic changes of carbon-nitrogen metabolism and stress responses. Plant Physiol **149:** 395–411

Westgate ME, Boyer JS (1984) Transpiration- and growth-induced water potentials in maize. Plant Physiol **74:** 882–889

Wobus U, Weber H (1999) Sugars as signal molecules in plant seed development. Biol Chem **380:** 937–944

Xiang L, Li Y, Rolland F, Van den Ende W (2011) Neutral invertase, hexokinase and mitochondrial ROS homeostasis: emerging links between sugar metabolism, sugar signaling and ascorbate synthesis. Plant Signal Behav **6:** 1567–1573

Xiao W, Sheen J, Jang JC (2000) The role of hexokinase in plant sugar signal transduction and growth and development. Plant Mol Biol **44:** 451–461

Xue GP, McIntyre CL, Glassop D, Shorter R (2008) Use of expression analysis to dissect alterations in carbohydrate metabolism in wheat leaves during drought stress. Plant Mol Biol **67:** 197–214

Xue HW, Chen X, Mei Y (2009) Function and regulation of phospholipid signalling in plants. Biochem J **421:** 145–156

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol **57:** 781–803

Yan HB, Jiang HW, Pan XX, Li MR, Chen YP, Wu GJ (2009) The gene encoding starch synthase IIc exists in maize and wheat. Plant Sci **176:** 51–57

Yu HG, Muszynski MG, Kelly Dawe R (1999) The maize homologue of the cell cycle checkpoint protein MAD2 reveals kinetochore substructure and contrasting mitotic and meiotic localization patterns. J Cell Biol **145:** 425–435

Yu LX, Setter TL (2003) Comparative transcriptional profiling of placenta and endosperm in developing maize kernels in response to water deficit. Plant Physiol **131:** 568–582

Zheng J, Zhao J, Tao Y, Wang J, Liu Y, Fu J, Jin Y, Gao P, Zhang J, Bai Y, et al (2004) Isolation and analysis of water stress induced genes in maize seedlings by subtractive PCR and cDNA macroarray. Plant Mol Biol **55:** 807–823

Zhuang Y, Ren G, Yue G, Li Z, Qu X, Hou G, Zhu Y, Zhang J (2007) Effects of water-deficit stress on the transcriptomes of developing immature ear and tassel in maize. Plant Cell Rep **26:** 2137–2147

Zinselmeier C, Jeong BR, Boyer JS (1999) Starch and the control of kernel number in maize at low water potentials. Plant Physiol **121:** 25–36

Downloaded from https://academic.oup.com/plphys/article/160/2/846/6109548 by U.S. Department of Justice user on 16 August 2022