# **Supporting Information**

### Liu et al. 10.1073/pnas.1301009110

#### SI Text

Plant Growth Conditions and Sample Collection. Seeds of Zea mays cv. White Crystal, a glutinous maize cultivar, were purchased from a local supplier and stored at room temperature. For germination, seeds were imbibed in distilled water at 6:00 PM by first shaking for 10 min at 200 rpm and then germinating on wet filter paper on Petri dishes in the greenhouse under natural sunlight conditions (approximately 6:00 AM to approximately 7:00 PM) during the summer. The first set of seeds was imbibed on 6/2/2011 and leaf tissue for RNA preparation was taken every 6 h for 2 d [up to the 48th hour (T48)]. A second set of seeds was imbibed at 6:00 PM on 7/24/2011 and leaf tissue for RNA preparation was taken at T54, T60, T66, and T72. The epicotyls were collected, and then coleoptiles were removed before the embryonic leaves were isolated. These isolated leaves were then stored in liquid nitrogen. The whole procedure was done in an hour. The maximum photosynthetic photon flux density around noon was ~1,600  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, and the day/night temperatures were 30–35 °C/ 25-28 °C, with a relative humidity of 60-70%. Samples of the embryonic leaves and the shoot apical meristem (SAM) were collected from dry seeds (T00) and germinating seeds every 6 h up to 72 h for leaf anatomic examination and for RNA isolation and transcriptomic analysis. For RNA isolation, ~200 plumules were dissected from the seeds and after removing the sheath tissue, the entire embryonic leaves and the SAM were stored in liquid N<sub>2</sub> until RNA extraction. Our tissue samples for transcriptomic analysis contained all of the four to five embryonic leaves in the maize seeds. As the first leaf is the largest, it should have the largest contribution to the transcriptomes. However, as new kranz structures (KSs) develop in these leaves, the transcriptomes are useful for hypothesizing the regulatory genes involved in KS development. The samples also contained SAM, but because SAM is small, its contribution to the transcriptomes may not be significant.

**Anatomical Studies.** For anatomical examination, samples of the embryonic leaves and SAM were fixed in 2.5% (wt/vol) glutaraldehyde and postfixed in 1% (wt/vol) OsO4, both in 0.1 M sodium phosphate buffer (1). After dehydration through an ethanol series, the samples were infiltrated and embedded in Spurr's resin. Semithin cross-sections (0.9  $\mu$ m) were cut from the base of embryonic leaves with an Ultracut E Microtome (Reichert-Jung) and stained with 0.1% (wt/vol) toluidine blue and 0.1% (wt/vol) borax for observation with a light microscope (Olympus).

RNA Extraction and Sequencing. Total RNA was extracted using TRIzol reagent (Invitrogen), and 1 µL TURBO DNase (Ambion) per 10 µg RNA was added and incubated for 30 min at 37 °C to remove traces of contaminating DNA; and RNA was subsequently purified by the phenol:chloroform procedure. The RNAs were quantified using Qubit RNA Pico reagent (Invitrogen) and assessed for purity and quality using NanoDrop (Thermo Scientific) and BioAnalyzer (Agilent). RNA-seq libraries were prepared using the Illumina Standard mRNA-seq library preparation kit (Illumina) based on the manufacture's protocol with the following modifications. Briefly, 15 µg of total RNA was subjected to two rounds of oligo-dT bead purification, fragmented for 2.75 min, and primed for first strand cDNA using 0.8× amount of random primer. After double strand cDNA synthesis and adaptor ligation, each sample was fractionated on 2% (wt/vol) Low Range Ultra Agarose (Bio-Rad) gels, from which the libraries of two different size ranges (~300 bp and

~400 bp) were excised. The purified libraries were then independently amplified by 15 cycles of PCR and cleaned up using AMPure XP beads (Beckman Agencourt). RNA-seq libraries were subjected to Illumina sequencing by paired-end 2\*101 nt (both ends with 101 nucleotides long) on a HiSeq2000 at the NGS Core Facility of Biodiversity Research Center in Academia Sinica, Taiwan. A total of 195–292 million pairs of raw reads was obtained from the two libraries (two lanes) for each time point sample; each pair of reads was counted as two reads. For each time point, ~390–584 million raw reads and 240–351 million mappable reads were obtained.

Data Processing and Analysis. Low-quality bases and reads were removed by three criteria: (i) the consecutive bases from the end of a read with a default low-quality score of 2 [phred score of 2 or Q2 (2)], (*ii*) the bases from the beginning of a read until all of the scores of the first 20 remaining bases were at least Q20 (the base call error rate of  $\sim 1\%$ ), and (iii) the trimmed reads with less than 60 remaining bases. (Phred score is a general metric for the accuracy of a sequencing platform (2). The Q2 indicator does not give a specific error rate, but rather indicates a specific portion of the read that should not be used in further analyses.) Each pair of reads was treated as two single reads. Then, all alignment and quantification processes followed the "alternative protocol B" in ref. 3). The processed reads were mapped to the maize genome (ZmB73\_RefGen\_v2) and the working gene set (ZmB73\_5a\_WGS, http://ftp.maizesequence.org/), using Tophat version 1.3.3 (http://tophat.cbcb.umd.edu/) and its embedded aligner Bowtie version 0.12.7 (http://bowtie-bio.sourceforge.net). Each read was aligned by the "-n" policy, and at most 10 hits were allowed. The expression level of each working gene set (WGS) gene was estimated in the reads per kilobase per million (RPKM) (4), using Cufflinks version 2.0.2 (http://cufflinks.cbcb.umd.edu/).

We selected only genes with RPKM  $\geq 1$  in two or more time points for further analysis. To compare the expression levels of the selected genes across all time points, we applied the upper quartile normalization procedure (5), which reduces the bias in RPKM values due to highly expressed genes. We used the transcriptome at T42 as the reference because its RPKMs were most evenly distributed among the 13 transcriptomes. The RPKM values at a time point were scaled by a constant factor to make the 75th percentile of the RPKM values for all expressed genes at that time point equal to the one at T42.

**Expression Profile Correlation and Clustering.** The clustering tool gCLUTO (Graphical Clustering Toolkit) was downloaded from http://glaros.dtc.umn.edu/gkhome/cluto/gcluto/overview. It includes several hierarchical clustering methods and we selected the repeated bisection method, which is a top-down hierarchical clustering algorithm that separates samples with the largest distance. We calculated the Pearson correlation coefficient (PCC) between each pair of expression profiles of the genes under study and used the PCC values to cluster the expression profiles. A scalar quantity, called the figure of merit (FOM) (6), was used to assess the quality of the clustering algorithm and to determine the optimal number of clusters.

To determine the functional enrichment for each cluster, we used the MapMan functional annotation (7). The annotated entries with the functional category name of "not assigned.unknown" were excluded in this study. We then used Fisher's exact test to examine whether a function was significantly over- or underrepresented in a selected cluster of genes against the set of all expressed genes with MapMan annotations.

Identification of Differentially Expressed Genes. We used the nonparametric method of Tarazona et al. (8) to identify differentially expressed genes (DEGs) between two samples. Here, we set the qvalue (differentially expression probability) in the method to be 0.7, because in our dataset genes with q > 0.7 have at least a 2.3-fold change in RPKM between the two samples. The test is stringent. For example, the number of DEGs between T06 and T12 was only 39, and because 30,255 genes (tests) were used, the probability of passing a test was only  $\sim 39/30,000 = 0.0013$ , which is much smaller than 0.01 (the 1% significance level). Thus, the test is conservative and can be used to identify DEGs between two time points.

- Sheue CR, et al. (2007) Bizonoplast, a unique chloroplast in the epidermal cells of microphylls in the shade plant Selaginella erythropus (Selaginellaceae). Am J Bot 94(12):1922–1929.
- Ewing B, Hillier L, Wendl MC, Green P (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8(3):175–185.
- Trapnell C, et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7(3):562–578.
- Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5(7):621–628.

The above analysis was between two time points. To find all genes differentially expressed during leaf development, we have developed the following method to make all pairwise comparisons between time points, which takes care of the problem of multiple testing. Suppose the probability of "success" in a single test is p and a total of N tests are made. We use the binomial expansion to calculate the probability of at least X successes in N tests. In the above, we have seen that p is only ~0.0013. To be conservative, let us take p = 0.005. Among the 13 time points there are 78 possible pairwise comparisons, that is n = 78. Then the probability to have at least X = 4 "successes" is only ~0.0001. Under this criterion, we find that 13,907 (46%) of the 30,255 expressed genes and 590 (47%) of the 1,238 expressed TF genes are differentially expressed during the 72-h time course.

- Bullard JH, Purdom E, Hansen KD, Dudoit S (2010) Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC Bioinformatics 11:94.
- Yeung KY, Haynor DR, Ruzzo WL (2001) Validating clustering for gene expression data. Bioinformatics 17(4):309–318.
- Thimm O, et al. (2004) MAPMAN: A user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J* 37(6):914–939.
- Tarazona S, García-Alcalde F, Dopazo J, Ferrer A, Conesa A (2011) Differential expression in RNA-seq: A matter of depth. *Genome Res* 21(12):2213–2223.

	тоо	T06	T12	T18	T24	T30	T36	T42	T48	T54	T60	T66	T72
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tip	0.02	0.02	0.02	0.02	0.03	0.03	0.04	0.04	0.04	0.03	0.03	0.04	0.04



Fig. S1. Comparison between mature leaf and embryonic leaf time course transcriptomes. Pearson's correlation coefficients (PCCs) between the four mature leaf transcriptomes of Li et al. (1), as indicated in the first column, and our time course transcriptomes, as indicated in the first row.

1. Li P, et al. (2010) The developmental dynamics of the maize leaf transcriptome. Nat Genet 42(12):1060–1067.

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**Fig. S2.** MapMan functional categories enriched in expressed genes at each time point. Enrichment tests conducted using (*A*) all expressed genes and (*B*) genes with top 10% or (*C*) top 1% RPKM values in each time point. The heatmap is color-coded based on the Z transformation of adjusted *P* values (1). Blue and red represent under- and overrepresented categories, respectively.

1. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc, B 57(1):289–300.

PNAS PNAS



Fig. S3. (Continued)

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Fig. S3. (Continued)

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Fig. S3. (Continued)

**DNAS** 

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Fig. S3. MapMan functional categories enriched in differentially expressed genes (DEGs). Enrichment tests with (A) type 1 DEGs, (B) type 2 DEGs, and (C) type 3 DEGs. (D) Clusters of all three types of DEGs. (E) Enrichment tests on each DEG cluster shown in D.

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	C01	Mapman Functional Categories	C01 C0	02 CO3	C04 0	:05 CO	6 C07 C	08 C09	C10 C1	1 C12	C13 C14	1C15C	16C17	C18 C	19 C20	C21 C2	2 C23	C24 C25	C26 C2	7 C28 C	:29 C30
		cell.vesicle transport	+			+		+	H	+	+		+		+			+		Ħ	+
	C02	development.late embryogenesis abundant																			
	C03	protein.degradation.ubiquitin.E3.RING	+			_		-		-	+	+	-		-	+	_	+	$\vdash$	++	+
		stress.abiotic.heat	+		$\square$			+	H			+	+		+			-	++	++	+
	<b>CO A</b>	TCA / org. transformation.other organic acid transformations.IDH	1																		
	C04	protein.degradation.ubiquitin.proteasom	_								+		_			$\vdash$			$\vdash$	$\square$	_
		minor CHO metabolism.myo-inositol.InsP Synthases amino acid metabolism.synthesis.aromatic aa.tryptophan.anthranilate synthase	+			+		-	$\vdash$		-		+		-	+	+	+	++	++	+
		mitochondrial electron transport / ATP synthesis.NADH-DH.localisation not clear											1								-
	C05	protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L21	$\square$																	Щ	
		protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L13	+			+		-	$\vdash$	+	+	+	+		+	+		+	$\vdash$	++	+
		protein-synulesis-nuosoniai protein-eukaryout-403 subunt-510 transport.potassium	+			+					+	+	+		+	+		-	++	++	+
	C06	mitochondrial electron transport / ATP synthesis.F1-ATPase	1																		
	C07	lipid metabolism.lipid degradation.lipases.triacylglycerol lipase	_			_		_					_		_	_		_	$\vdash$		_
		mitochondrial electron transport / ATP synthesis.cytochrome c oxidase protein.synthesis.ribosomal protein.prokarvotic.chloroplast.305 subunit.S12	+			+		+	$\vdash$	+	-	+	+		+	+	-	+	++	++	+
	C08	protein.synthesis.ribosomal protein.prokaryotic.mitochondrion.30S subunit.S7	+							t		Ħ	+		1	$\uparrow$		+	Ħ	++	+
	C09	protein.synthesis.ribosomal protein.prokaryotic.mitochondrion.50S subunit.L16	T																	Щ	
	C10	PS.lightreaction.cytochrome b6/f	_			-		+	$\square$		_				-	$\vdash$		_	$\vdash$		_
	010	RNA.regulation of transcription.Global transcription factor group	+			-		+					+		-		+	+	+	++	+
	C11	RNA.regulation of transcription.Bromodomain proteins																			
	C12	secondary metabolism.flavonoids.anthocyanins.anthocyanin 5-aromatic acyltransferase	+								+	$\square$	-			$\vdash$	_	_	$\square$	$\square$	_
	C13	RNA.regulation of transcription.AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family protein degradation ubiquitin F3 SCF EROX	+			+	$\left  \right $	+	$\vdash$	+	+	+	+		+	+		+	++	++	+
	C14	misc.UDP glucosyl and glucoronyl transferases	+					+	H	+	+		-		+			-	Ħ	++	+
	C14	signalling.calcium	I																		
	C15	signalling.receptor kinases.Catharanthus roseus-like RLK1	+			-		+	$\left  \right $	-	+		_		-	$\vdash$	_	_	$\vdash$		_
	C16	RNA.regulation of transcription.HB,Homeobox transcription factor family	+		$\square$	+		+		+	+	+	+		+	+		+	H	++	+
		RNA.regulation of transcription.ARF, Auxin Response Factor family	1																		
	C17	RNA.regulation of transcription.ABI3/VP1-related B3-domain-containing transcription factor family	_			_		+	$\square$		+		_		-	$\vdash$		+	$\square$	++	+
	C18	protein.targeting.nucleus development.unspecified	+			+		+	$\left  \right $	+	+	+	+		-		+	+	++	++	+
		minor CHO metabolism.callose																			
	C19	protein.degradation.ubiquitin.ubiquitin protease	_			_		+			+		_		_			_	$\vdash$		_
		RNA.processing.degradation dicer RNA regulation of transcription Chromatin Remodeling Factors	+		$\vdash$	+		+	$\vdash$	+	+	++	+		+	-	+	+	$\vdash$	++	+
	C20	RNA.regulation of transcription.PHD finger transcription factor	+					1		t	+	Ħ	+		1			+	Ħ	++	+
		RNA.transcription																			
	C21	signalling.light	+			-		+	$\left  \right $	-	+		+		-	-		+	$\vdash$	++	+
		DNA.synthesis/chromatin structure	+			+		+	$\left  \right $	+			+		+		-		$\square$	++	+
	C22	RNA.regulation of transcription.MYB domain transcription factor family																			
		DNA.unspecified	+					-		-	+	+	+		-	+	_	_	H	++	+
	C23	misc.beta 1,3 glucan hydrolases	+					+		+	-		+		+	-			╋	++	+
		DNA.synthesis/chromatin structure.histone	1																		
	C24	cell.cycle	_			_		_			+		_			$\vdash$			$\square$		_
		RNA.regulation of transcription.Nucleosome/chromatin assembly factor group mitochondrial electron transport / ATP synthesis.cvtochrome c reductase	+			+		+	$\vdash$	+	+	+	+		+	+	-		$\vdash$	++	+
		protein.synthesis.ribosomal protein.eukaryotic.40S subunit.S23	1									Ħ			t	d t					
	C25	protein.targeting.mitochondria	_					_			_		_		_	$\vdash$		_			_
		stress.biotic RNA regulation of transcription Psudo ABR transcription factor family	+			+		+	$\vdash$	-	+	+	+		+	+	+	+	┢╋╋	4	_
	C26	cell.cycle.peptidylprolyl isomerase	+					+			-		+		+			-			-
		protein.folding	1								1	$\square$						1		$\square$	
	C27	tetrapyrrole synthesis.uroporphyrinogen III synthase	+		$\left  \right $	-	$\left  \right $	+	$\mathbb{H}$	+	+	+	+	$\left  \right $	+	+	+	+	++	+	+
	C28	vensport.p- and v-A Pases.n+-transporting tWO-Sector ATPase PS.lightreaction.photosystem II.LHC-II	+	+	$\square$		+	+	$\mathbb{H}$	+	+	+	+	+	+	+	+	+	++	++	+
	C29	PS.lightreaction.photosystem II.PSII polypeptide subunits	1										T						$\square$		
		PS.lightreaction.photosystem LPSI polypeptide subunits					$\square$		$\parallel$		+	$\parallel$	_			+	+	+	$\vdash$	+	
	C30	HS.lightreaction.other electron carrier (ox/red).ferredoxin misc.misc2	+	-		+	$\left  \right $	+	$\parallel$	+	+	+	+	+	+	+	+	+	++	+	+
the second se		1	_	-	-	_				-	_		_		-	_		-			_

Fig. S4. (Continued)

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**Fig. S4.** MapMan functional categories enriched in genes of various coexpression gene clusters. (*A*) Expression profiles of genes sorted according to cluster orders. (*B*) Enrichment tests of detailed overrepresented functional categories on each cluster as shown in *A*. (*C*) Expression profiles of genes in selected clusters. (*D*) Enrichment tests of both over- and underrepresented high-level categories on each cluster as shown in *C*. (*E*) Enrichment tests of both over- and underrepresented high-level categories on each cluster as shown in *C*.



Fig. S5. Maize transcription factors likely regulating vascular development. (A) The current known components and relationships of vascular development genes in Arabidopsis thaliana. (B) Expression patterns of putative maize orthologs of A. thaliana vascular development genes.

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		Quality fi	ltering	Mappable	Mappable read				
Sample	Library length, bp	Total reads	Rate, %	Total	Rate, %				
T00_ST-DA01	300	218,477,277	75.9	277,488,186	76.6				
	400	143,975,388	86.8						
T06_ST-DA02	300	213,618,797	74.8	255,413,163	71.7				
	400	142,589,424	85.7						
T12_ST-DA03	300	206,947,451	74.6	275,874,436	75.9				
	400	156,344,245	82.1						
T18_ST-DA04	300	210,270,688	76.5	279,889,765	76.4				
	400	155,975,570	84.0						
T24_ST-DA05	300	190,425,691	78.2	294,733,426	78.8				
	400	183,789,389	79.9						
T30_ST-DA06	300	195,697,940	80.2	271,280,993	77.9				
	400	152,421,099	85.6						
T36_ST-DA07	300	215,565,717	79.9	308,395,082	80.0				
	400	169,797,300	84.6						
T42_ST-DA08	300	153,005,724	85.2	242,223,816	72.2				
	400	182,552,562	81.7						
T48_ST-DA09	300	156,382,336	83.7	240,264,229	73.1				
	400	172,108,685	84.8						
T54_ST-DB05	300	222,228,743	76.8	340,039,005	81.2				
	400	196,448,375	82.1						
T60_ST-DB06	300	236,437,704	76.3	351,089,370	80.6				
	400	199,181,671	82.3						
T66_ST-DB07	300	237,623,498	74.6	339,580,019	75.6				
	400	211,538,295	79.9						
T72_ST-DB08	300	208,071,129	80.7	320,303,598	75.3				
	400	217,502,039	76.5						

Table S1. Read count statistics of the deep sequencing data for the 13 time points studied

#### Table S2. Numbers of annotated genes and expressed features

		No. of expressed features								
A/E	CDS	TF	Pseudo	TE						
A 63,36		2,070	17,344	29,024						
Е	30,255	1,238	3,040	1,986						
А	39,441	2068	95	53						
Е	23,900	1,237	95	53						
	A/E A E A E	A/E CDS A 63,363 E 30,255 A 39,441 E 23,900	No. of expression           A/E         CDS         TF           A         63,363         2,070           E         30,255         1,238           A         39,441         2068           E         23,900         1,237	A/E         CDS         TF         Pseudo           A         63,363         2,070         17,344           E         30,255         1,238         3,040           A         39,441         2068         95           E         23,900         1,237         95						

A, all; CDS, protein coding gene; E, expressed (RPKM  $\geq$ 1 in more than one time point); FGS, filtered gene set; pseudo, pseudogene; TE, transposable element; TF, transcription factor; WGS, working gene set.

## **Other Supporting Information Files**

Dataset S1 (XLSX) Dataset S2 (XLSX)

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