

Anatomical and transcriptional dynamics of maize embryonic leaves during seed germination

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Our anatomical analysis revealed that a dry maize seed contains four to five embryonic leaves at different developmental stages. Rudimentary kranz structure (KS) is apparent in the first leaf with a substantial density, but its density decreases toward younger leaves. Upon imbibition, leaf expansion occurs rapidly with new KSs initiated from the palisade-like ground meristem cells in the middle of the leaf. In parallel to the anatomical analysis, we obtained the time course transcriptomes for the embryonic leaves in dry and imbibed seeds every 6 h up to hour 72. Over this time course, the embryonic leaves exhibit transcripts of 30,255 genes at a level that can be regarded as “expressed.” In dry seeds, ~25,500 genes are expressed, showing functional enrichment in transcription, RNA processing, protein synthesis, primary metabolic pathways, and calcium transport. During the 72-h time course, ~13,900 genes, including 590 transcription factor genes, are differentially expressed. Indeed, by 30 h postimbibition, ~2,200 genes expressed in dry seeds are already down-regulated, and ~2,000 are up-regulated. Moreover, the top 1% expressed genes at 54 h or later are very different from those before 30 h, reflecting important developmental and physiological transitions. Interestingly, clusters of genes involved in hormone metabolism, signaling, and responses are differentially expressed at various time points and TF gene expression is also modular and stage specific. Our dataset provides an opportunity for hypothesizing the timing of regulatory actions, particularly in the context of KS development.

plant leaf development | plant hormones | gene expression profiling

Maize, a well-studied crop, has been used as a model plant for C₄ photosynthesis study, as its leaves possess the kranz structure (KS) for efficient photosynthesis. However, how its leaves develop from seed following imbibition has not been well studied. In particular, it is unclear how KS forms during leaf development. Using the next generation sequencing technology, Li et al. (1) studied the leaf transcriptomes of four regions of 9-d-old third maize leaves: the base, the tip, and two middle regions of the leaf, representing different leaf developmental stages, with the base being the youngest. The data revealed a dynamic transcriptome profile, showing different transcripts enriched in different regions and providing a preliminary view of molecular changes during maize leaf development. However, as the leaf base already exhibits distinct KS, it is not early enough to represent the early leaf development when KS begins to form. Indeed, our anatomical study reveals that KS already exists in a rudimentary form in the first two embryonic leaves of maize dry seeds, and the embryonic leaves develop rapidly after seed imbibition (see below). To correlate the transcriptomic dynamics with the KS development during seed germination, we have obtained the time course transcriptomes of embryonic leaves at every 6 h, starting from dry seeds to hour 72 postimbibition. This set of data provides a clear picture of the transcriptional dynamics of genes for the early leaf development during maize seed germination. Moreover, we use the data to infer the succession

of biological processes during this period. Finally, we examine what hormone-related genes and transcriptional regulators are differentially expressed in the embryonic leaves over time, providing a global view of the molecular changes underlying their anatomical and physiological transitions during seed germination.

Results and Discussion

Anatomical Changes During Early Leaf Development. Fig. 1*A* shows the morphological changes in maize seeds during the first 72 h of imbibition; the tissue swells over time until the appearance of coleoptile at the 72nd hour (T72). A dry maize seed contains four to five embryonic leaves (Fig. 1*B*). The first leaf already has many well-differentiated rudimentary KS units separated by one to two ground meristem (GM) cells in the middle (median) layer of leaf primodium. The second leaf is less developed, but has recognizable KS units. Upon imbibition, all embryonic leaves expand within 12 h (Fig. 1*C*). Compared with the first embryonic leaf, the younger leaves contain fewer veins (KS units), frequently separated by more than five palisade-like median ground meristem (mGM) cells. During the next 2 d, the vein density rapidly increases (Fig. 1*D* and *E*).

The most significant feature of the maize embryonic leaves, except for the main vein regions, is that they consist of five layers of distinct cell types in transverse section (Fig. 1*F*): two layers of protoderm (P) and three layers of GM cells, with the mGM cell layer located in the middle of the leaf and sandwiched by two layers of round-shaped ground meristem (sGM) cells. The two layers of protoderm form the adaxial and abaxial epidermal cell layers, whereas contiguous mGM cells differentiate into vascular tissue, bundle sheath (BS) cells, and mesophyll (M) cells. Clearly, all KS units of the minor veins arise from the mGM cells, initially with a simple formation of three to four contiguous cells with two cells sandwiching one or two smaller cells. Fig. 1*G–K* shows the progressive changes in KS or vein development. The sGM cells next to the protodermal cell layers give rise to the subprotodermal mesophyll (sm) cells (Fig. 1*K*) separating the veins and the epidermal cells in mature leaves. KS units at different developmental stages can be found in all embryonic leaves, especially in the younger leaves. In initiating new KS units the progenitor mGM cells begin to divide and

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a slightly faster decrease in r than the top 1% of genes until T48 but a slower rate of decrease after T48 (Fig. 2B, Upper half). TF genes show a slower rate of decrease in r initially than other genes but a faster rate after T54 (Fig. 2B, Lower half).

As mentioned above, an earlier study obtained the transcriptomes of four regions of 9-d-old third maize leaves (1). No significant correlation exists between their transcriptomes and ours, except for the leaf base (the youngest region), and the maximum correlation is only 0.64, which occurs at T60 (Fig. S1). Thus, the base of a 9-d-old maize leaf may not represent the very early leaf development and our time course data provide a unique global view of gene expression over maize early leaf development.

Genes Expressed in the Embryonic Leaves of Dry Seeds. In dry seeds, transcripts for 20,603 (52%) FGS and 25,401 (40%) WGS genes are already present with RPKM ≥ 1 . These transcripts may jump-start the maize leaf development upon imbibition. To assess the roles of these stored transcripts, we examine MapMan functional categories containing an over- or underrepresented number of expressed genes at T00 (Fig. S24).

Three types of genes are overrepresented at T00. The first type includes genes involved in transcription, RNA processing, protein synthesis, and primary metabolic pathways, including carbohydrate biosynthesis, the tricarboxylic acid (TCA) cycle, mitochondrial electron transport, and fatty acid, nucleotide, and amino acid biosynthesis. These are likely transcripts stored in the embryonic leaves for meeting the demands for rapid growth upon imbibition. Similarly, cell division genes are overrepresented, potentially contributing to rapid cell proliferation. The second type includes genes involved in calcium transport, likely reflecting the role of calcium in removing the inhibitory effect of abscisic acid (ABA) during germination (4). The third type includes stress genes, whose expression may be caused by dehydration and osmotic stress during seed maturation. Interestingly, genes involved in abiotic stress are overrepresented but those in biotic stress are underrepresented in the quiescent leaves of the dormant seeds.

Some of the above categories are enriched not only in dry seeds but also throughout the time course (Fig. S24). These categories include primary metabolic pathways, such as the TCA cycle, some components of mitochondrial electron transport, and biosynthesis of amino acids, proteins, and nucleotides, likely for the need of energy and building blocks for early leaf development. On the other hand, genes in some functional categories are underrepresented, including biotic stress, cell wall modification and degradation, and secondary metabolism.

Functional Transition Postimbibition. Although some functional categories contain consistently over- or underrepresented numbers of expressed genes over the entire time course, only 852 (2.8%) genes are expressed at similar levels (coefficient of variation <0.2) during the first 72 h of imbibition and 71 of the 852 genes show more than twofold changes in RPKM value during the 72 h. Thus, most genes are up- or down-regulated to various degrees over the 72-h period (Fig. 3A). In addition, some biological processes are enriched in a time-point-specific manner (Fig. S24). For example, genes involved in cell division and calcium transport are overrepresented until T30 (Fig. S24). Genes of E3 ligases involved in ubiquitin-mediated protein degradation are particularly prominent at T12–T18, indicating the elevated need for protein turnover. In addition, chromatin structural genes tend to be expressed from T30 to T72. Moreover, many categories have underrepresented numbers of expressed genes in a time-point-specific manner. For example, ethylene signal transduction genes exhibit reduced expression during T54–T72, presumably reflecting a reduced influence of ethylene at this stage.

To assess functional transition due to highly expressed genes, we conducted enrichment tests on genes in the top 10% or 1% (Fig. S2B and C). Consistent with the above analysis using all genes, categories containing, for example, ATP and protein synthesis or abiotic stress genes are enriched. However, the analysis of the top 10% of genes reveals more categories that are time-

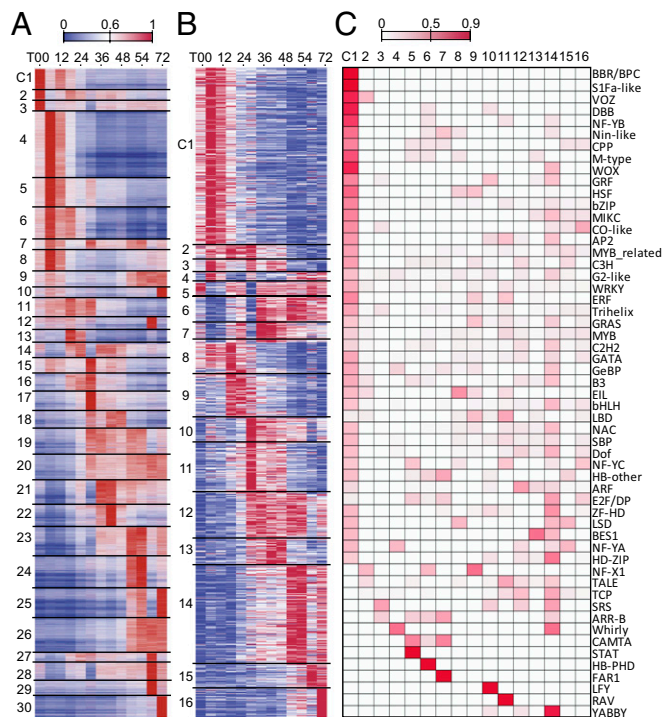


Fig. 3. Functional transition and gene expression pattern over the time course. (A) Expression patterns of 30 coexpression modules (C01–C30), ordered according to the time point of their expression peak. For each gene, the value shown is the RPKM value normalized by the maximum value of all RPKM values of the gene in question over all time points. (B) Normalized expression levels of 1,238 TF genes sorted into 16 expression modules (clusters) (C1–16). (C) Proportion of TFs in a family that are members of a cluster.

point specific. For example, a major class of plant cell wall proteins, hydroxyproline-rich glycoproteins (HRGPs), which are involved in growth and cell wall development, are enriched at T00 and T12. One HRGP (GRMZM2G168651) has been shown to be expressed in maize meristem and vascular tip (5), consistent with the appearance of vascular tissues in embryonic leaves (Fig. 1). Another example is that, at T54, categories containing ribosomal proteins, ATPase transporters, and peroxiredoxins are overrepresented. Peroxiredoxins are targeted to chloroplasts to protect the photosynthetic membrane (6) by scavenging toxic peroxides produced during electron transport. Finally, at T72 genes involved in brassinosteroid (BR) metabolism are overrepresented, suggesting the rapid formation of vascular tissues at this stage, as BR promotes cell expansion and vascular differentiation (7, 8).

Differential Gene Expression Between Time Points. We identify three types of differentially expressed genes (DEGs) between time points (Table 1). Type 1 DEGs are identified by comparing the transcriptome at a time point against its preceding one, signifying the transition in gene function in 6 h. From T00 to T06, the transcript levels of 229 genes (including 7 TF genes) are decreased, whereas from T12 to T18, 481 genes (including 22 TF genes) are up-regulated. Notably, a sharp increase occurs in down-regulated DEGs from T24 to T30 (1,136 genes, including 28 TF genes) and in up-regulated DEGs from T30 to T36 (1,585 genes, including 50 TF genes). These and other large changes in DEG numbers suggest major developmental or physiological transitions. Interestingly, a group of genes down-regulated at T30 is up-regulated at T36, including RNA regulation of transcription (including many TFs), chromatin remodeling factors, histone acetyltransferases, DNA synthesis/chromatin structure, and cell cycle/division (Fig. S3A, D, and E).

Table 1. Numbers of differentially expressed genes and TFs between two time points

Time (T)	All genes (TF genes): Up-regulated/down-regulated		
	T vs. T-6 h	T vs. T00	New DEGs
T06	45 (0)/229 (7)	45 (0)/229 (7)	45 (0)/229 (7)
T12	21 (0)/18 (0)	51 (2)/81 (0)	35 (2)/21 (0)
T18	481 (22)/75 (0)	466 (35)/43 (0)	427 (34)/38 (0)
T24	225 (5)/75 (5)	1182 (60)/552 (13)	824 (34)/510 (13)
T30	136 (16)/1136 (28)	2035 (152)/2242 (82)	1142 (96)/1638 (64)
T36	1585 (50)/189 (9)	2555 (133)/2423 (81)	877 (24)/1015 (32)
T42	20 (0)/32 (0)	2402 (130)/2404 (88)	280 (12)/225 (10)
T48	143 (2)/16 (0)	2379 (130)/2089 (98)	305 (9)/119 (15)
T54	343 (4)/466 (47)	3700 (148)/2961 (157)	1351 (46)/686 (50)
T60	46 (3)/64 (2)	3980 (171)/3340 (171)	523 (23)/371 (20)
T66	718 (17)/705 (17)	2981 (91)/2602 (140)	464 (6)/310 (18)
T72	646 (14)/615 (33)	3731 (152)/3042 (186)	424 (20)/223 (20)

New DEGs, differentially expressed genes not observed until that time point; T-6 h, time point 6 h earlier than time T, that is, the time point preceding T.

Type 2 DEGs are defined by comparisons between a time point and T00, representing genes that are up- and/or down-regulated postimbibition (Table 1 and Fig. S3B); some type 2 DEGs overlap with type 1 DEGs. Many genes become up-regulated 18 h after imbibition and many functional categories are enriched in type 2 DEGs at various time points, including arabinogalactan proteins (AGPs) and cell wall proteins at T18, DNA synthesis/chromatin structure at T24, cell wall cellulose synthesis at T30, fatty acid metabolism at T36, BR signal transduction at T42, the YABBY TF family at T48, and nucleotide metabolism at T54. At T72 genes for nitrate metabolism and chloroplast targeting are up-regulated (Fig. S3B). In *Arabidopsis*, YABBY members are responsible for specifying the leaf adaxial identity (9). On the other hand, genes in a number of categories are down-regulated such as RNA helicase at T06, late embryogenesis abundant (LEA) proteins at T24, biodegradation of xenobiotics at T30 and T36, ATP synthesis at T36, and many TF families [AP2 (apetala 2)/ERF (ethylene-responsive factor), CO (constans)-like, EIN3 (ethylene insensitive 3)-like and bZIP (basic leucine zipper)] and ubiquitin E3 at T48.

The third type includes genes not differentially expressed until the indicated time points; they are a subset of type 2 DEGs. Type 3 DEGs potentially signify the onset/termination of developmental and/or physiological processes at a particular stage (Fig. S3C). An example is the differential regulation of cell wall proteins (Dataset S1). AGPs and leucine-rich repeat proteins (LRRs) are up-regulated at T18, T30, or T60; cellulose synthases are up-regulated at T18 or T66; cell wall modification proteins are up-regulated at T18, T24, or T54; pectin esterases, which are involved in cell wall modification and breakage, are up-regulated at T24 or T30; and cell wall degradation proteins (e.g., cellulases and pectate lyases) are up-regulated at T24, T30, or T36, which may be important for subsequent cell division (Dataset S1). Differential regulation of genes of the same function/process reveals how distinct components are used at different stages during leaf development.

Overall, the total number of DEGs over the entire time course is estimated to be 13,907 if all pairwise comparisons are included. Thus, close to half (46%) of the 30,255 expressed WGS genes may be regarded as differentially expressed during the 72-h time course. The corresponding proportion for expressed TF genes is 47% (590/1,238).

Coexpression Modules. Using hierarchical clustering, we classify the expressed WGS genes into 30 coexpression modules, each of which contains genes with highly similar expression patterns (Fig. 3A and Fig. S4A). In the following analysis, we select 20 modules

(Fig. S4C) that together contain ~83% (12,716) of the 15,372 expressed genes with MapMan annotation (Fig. S4B and D). With some exceptions, the clusters can be sorted according to the time point the peak expression occurs. This clustering analysis allows us to aggregate genes over multiple time points, providing another view of functional transitions along the early maize leaf development.

The 20 selected modules can be roughly divided into early (T00–T12), middle (T18–T48), and late (T54–T72) stages (Fig. 3A and Fig. S4C and D). The early stage, best represented by modules C01, C04–C06, and C08, coincides with the transition from seed dormancy to germination, which involves extensive physiological changes (10). In C01, the expression level is high at T00 and T12 but becomes lower after T18. C01 includes genes related to lipid metabolism (Fig. 3A and Fig. S4D). These transcripts are likely mRNAs stored for the very early stage of leaf development during germination. In C04–C06, and C08, gene expression peaks at T06 but different modules have distinct profiles after the peak. ABA and ethylene-related genes belong to these modules (Fig. S4D and E), reflecting the roles of these hormones during early leaf development (11). Genes related to protein degradation and synthesis are also enriched in these modules, suggesting that protein metabolism is essential for the development of embryonic leaves. For example, functional categories related to ubiquitin E3 SCF (Skp1-Cullin-F-box protein) and RING (really interesting new gene) domain proteins are overrepresented in C05 (Fig. S4B), perhaps because several E3 ligases are major components of hormone signaling (12).

The middle stage (T18–T48), represented by C13, C14, C16–C21, and C23, is typified by the up-regulation of auxin and gibberellic acid (GA) related genes, which are involved in cell division and differentiation, and genes in transcriptional regulation (Fig. S4D and E). This stage coincides with the rapid embryonic leaf and KS development in the first 2 d of imbibition (Fig. 1C and D). Also, genes in mitochondrial electron transport, ATP synthesis, and glycolysis are overrepresented (Fig. S4D), indicating a heightened energy consumption, presumably taking place following digestion of endosperm. Meanwhile, the elevated transcription levels of transcriptional regulatory genes indicate the onset of multiple regulatory cascades responsible for various aspects of leaf development.

At the late stage (T54–T72), which includes C23–C25, and C30, jasmonate (JA) metabolic genes are up-regulated (Fig. S4E). C30 includes genes involved in photosynthesis, e.g., the RuBisCO (ribulose-1,5-bisphosphate carboxylase oxygenase) small subunit gene. Note that at T66 the entire shoot apex covered by sheath could already be seen outside the seed and became light green (Fig. 1A). Transcripts for photosynthesis genes start to accumulate at T48–T54 and become highly enriched at T72, likely signifying the onset of heightened photosynthetic activities. This is consistent with the analysis of type 1 DEGs, where genes involved in photosynthesis start to be up-regulated at T48 (Fig. S3A).

Developmental Transition of Hormonal Functions. During the first 72 h of leaf development, 431 expressed genes are relevant to the action of eight hormones: ABA, auxin, BR, cytokinin (CK), ethylene, GA, JA, and salicylic acid (SA) (Fig. 4). For each hormone class, genes are classified into three types: (i) synthesis-degradation, (ii) signal transduction, and (iii) induced-regulated-responsive-activated (Fig. 4 and Dataset S2). Most of these hormone-related genes are expressed in a time-point/range-specific manner (Fig. 4). A relatively small number of hormone-related genes have transcripts in the embryonic leaves of dry seeds (T00), including aldehyde oxidase 1 and 4 involved in seed ABA biosynthesis and maize orthologs of *GA3* (gibberellin A3) involved in the GA biosynthesis (Dataset S2).

Many synthesis-degradation (type 1) genes are highly expressed at T06 and continue to express until T12 or T18 (Fig. 4 and Dataset S2). The highly expressed hormone biosynthesis genes include maize orthologs of *NCED3* (9-cis-epoxycarotenoid dioxygenase 3) (ABA), *GAI* (gibberellin A1), and *GA2OX1* (GA 2-

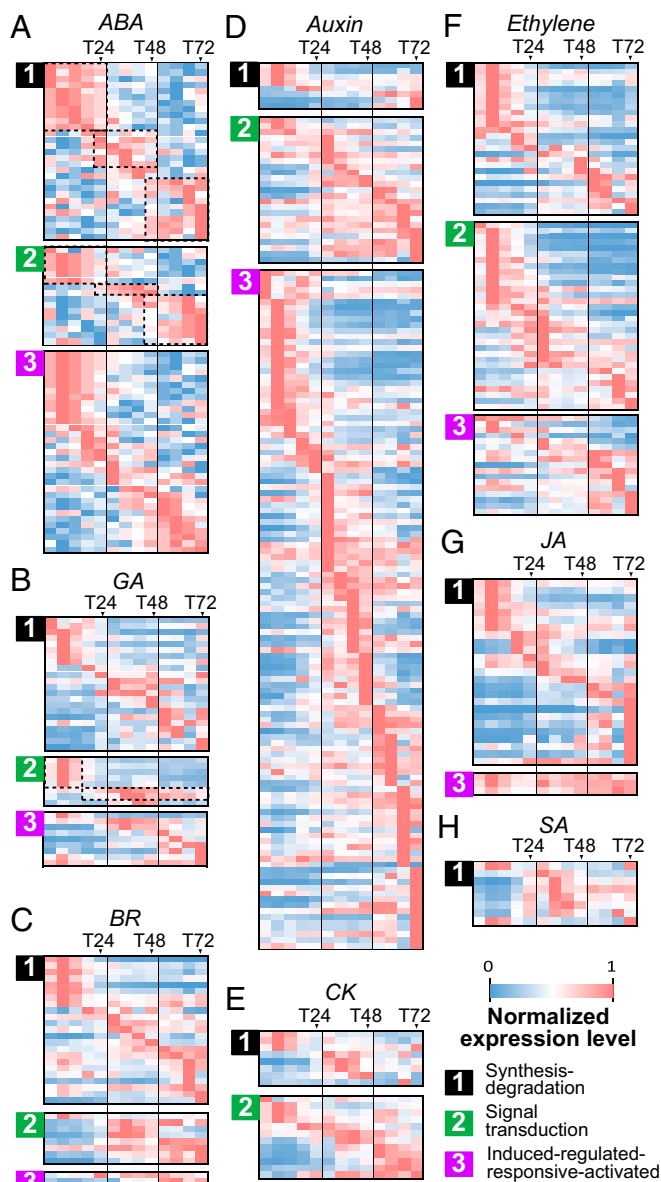


Fig. 4. Expression patterns of hormone-related genes. Normalized expression levels of genes related to (A) abscisic acid (ABA), (B) gibberellic acid (GA), (C) brassinolide (BR), (D) auxin, (E) cytokinin (CK), (F) ethylene, (G) jasmonic acid (JA), and (H) salicylic acid (SA) are shown. Normalized expression level is calculated as in Fig. 3. For each hormone, its related genes are divided into three functional categories: (i) synthesis-degradation, (ii) signal transduction, and (iii) induced-regulated-responsive-activated.

oxidase 1) (GA), *DET2* (deetiolated2) (BR), and *SRG1* (senescence-related gene 1) (ethylene) (Dataset S2), consistent with their important roles in breaking seed dormancy (13). In addition to the early time points, several ABA-, BR-, JA-, and SA-related type 1 genes are also highly expressed at T66 or T72 (Fig. 4A, C, G, and H). Examples of BR response genes include maize orthologs of *Arabidopsis thaliana* *CYP51G1* (cytochrome P450 51G1) and *DWF1* (dwarf 1), which regulate cell elongation, and *SMT2* (sterol methyltransferases 2), which regulates vascular differentiation (Dataset S2). For JA synthesis, many lipoxygenase family genes are highly expressed at T72, which may be required for wound response (Dataset S2). Apparently, the surge in expression of these genes in the embryonic leaves at the later stage

of germination reflects the active differentiation of vascular tissues and the onset of plant defense mechanisms.

Type 2 genes include those relevant to hormone perception and downstream signaling, e.g., protein kinases and transcriptional regulators. For ABA, GA, BR, and ethylene, there are distinct coexpression modules among the type 2 genes (Fig. 4, type 2). For example, for both type 1 and type 2 ABA genes, there are three distinct modules: from T00 to T24, from T24 to T48, and from T48 to T72 (Fig. 4A, dotted rectangles). The T00–T24 module includes maize *VPI* (viviparous1) and the maize ortholog of *ABI5* (ABA-insensitive 5), regulating the transition from germination to vegetative growth (Dataset S2), and *ABF2* (ABRE-binding factor 2) up-regulated for ABA signaling under water stress (14). The T48–T72 module includes the maize ortholog of *LEC2* (leafy cotyledon2) that functions in embryo development (Dataset S2). Type 2 modules also include GA genes (Fig. 4B, type 2). The T00–T12 module includes a GA receptor and a GA signaling repressor, the maize orthologs of *GID1c* (GA insensitive dwarf 1c) and *GAI* (GA-insensitive), which are involved in seed germination and stem elongation (Dataset S2). The second type 2 GA module (T18–T72) contains another two maize *GAI* and *SPY* (spindly) orthologs with a peak expression at T36–T42 (Dataset S2). *SPY* is related to suppression of GA response, while promoting CK signaling (15). Thus, T18 may mark a transition point between GA and CK functions, from cell elongation to differentiation, coinciding with the rapid maturation of existing KSs and initiation of new KSs after T18 (Fig. 1C). Another type 2 gene that signifies interactions between hormones is the maize orthologs of the ethylene receptor *ETR1* (ethylene receptor1), known to inhibit ABA signaling (Dataset S2). There are seven putative orthologs of CK receptors *AHK2* (arabidopsis histidine kinase 2), *AHK3*, and *AHK4*, but only one putative ortholog of auxin receptor *TIR1* (transport inhibitor response 1) in maize. These *AHK* genes are important regulators of shoot vascular tissue development in *A. thaliana* (16) and most of their transcripts start to accumulate after T12. In contrast, *TIR1* expression first peaks at T06, followed by a reduction, and peaks again between T30 and T72. Interestingly, the second peak expression coincides with the maize orthologs of *BIG* (formerly *DOC1/TIR3/UMB1/ASA1*), *PIN1* (pin-formed 1), and *PIN4*, all of which play important roles in auxin transport and vein patterning (17). These observations are consistent with the vein density increase after T24 (Fig. 1C and D) and suggest that leaf vein or KS development may be mediated by relocation of the hormones via the action of these receptor and transport genes before T30.

One may expect that the type 3 genes that are induced by the same hormones as those for types 1 and 2 genes would have similar ranges of expression (Fig. 4, type 3). Indeed, two maize orthologs of ABA-responsive genes *EM1* (late embryogenesis abundant 1) and *EM6* required for buffering water loss during normal seed development (18) exhibit the highest expression levels from T00 to T24 (Dataset S2). Another example is the maize orthologs of ABA-induced *HVA22* (HVA22 domain protein), which regulates seed germination by inhibiting GA-mediated vacuolation, are highly expressed at T06, but much lower thereafter (Dataset S2).

Expression Dynamics of TFs. The 1,238 expressed TF genes are classified into 16 coexpression modules or clusters, which are ordered by their expression peaks (Fig. 3B). Almost all TF genes have relatively narrow windows of expression (Fig. 3B), suggesting regulatory successions that underlie physiological and developmental transitions.

C1, the largest cluster, contains 328 TFs that have high expression levels during the first 24 h, followed by a gradual decrease to a very low level at T72 (Fig. 3B). Most TF families have members in C1 (Fig. 3C), including *VPI*, which is responsible for the establishment of seed dormancy (19), and *Opaque2* (O2) heterodimerizing protein 1, which together with O2 regulates the expression of zein storage protein genes (20). Similar to C1, C8–C11 and C13–C16 have single expression peaks at different time points, indicative of regulatory succession. For example, *MWP1*

(milkweed pod1), which is in C10, is responsible for adaxial-abaxial leaf surface patterning (21). Because C10 has a peak at T30, the polarity of newly emerged leaves may be established at the second day postimbibition. Another example is *Golden 2* (*G2*), which is involved in maize leaf BS chloroplast development (22). *G2* (*Zmglk2*) transcript is present in dry seeds and is in C14, which peaks at T54, indicating the onset of BS chloroplast development after T48, consistent with the inference from the DEG (Fig. S3 A–C) and cluster (Fig. S3 D and E) analyses. In contrast, the transcript of *ZmGkl1*, which is responsible for M chloroplast development in maize leaves (23), remains low until T54 (C15). Thus, BS chloroplasts may develop before M chloroplasts. There are other TFs with known functions in these clusters but their roles in early leaf development are not clear. For example, *ZFL2* (*zea floricaula leafy2*) in C10 is known to regulate floral transition and flower development (24) but it is not clear why it is expressed so early postimbibition.

Although the role of most TFs in early leaf development is not known, their orthologs in *A. thaliana* provide hints on their functions. Take vascular development as an example. Several key *A. thaliana* regulators have roles in establishing and maintaining procambial and cambial cell populations and for further specification and differentiation into distinct cell types within xylem or phloem (25). For example, *A. thaliana* *BDL* (bodenlos), an AUX/IAA family protein, suppresses *MP* (monopteros), which is important for preprocambial development and procambium differentiation in embryo (26). Expression of *BDL* ortholog GRMZM2G142768 peaks at T06 and gradually declines thereafter (Fig. S5). In contrast, the expression levels of maize *MP* orthologs (GRMZM2G034840 and GRMZM2G086949) start arising at T24 when *BDL* starts to drop. This contrasting pattern of expression suggests that the inhibition for vascular development is released at ~T24 because *BDL* is down-regulated by an elevated level of auxin (27). This is consistent with elevated levels of the maize orthologs of *TIR1* and *PIN1* as discussed above. In addition, the levels of genes involved in vein formation begin to rise at T24 (Fig. S5). Thus, our time course data allows hypotheses to be generated and tested regarding when these TFs may function over the course of early maize leaf development.

Concluding Remarks

Our tissue samples for transcriptomic analysis contained all of the embryonic leaves in the seeds. As the first leaf is the largest, it should have the largest contribution to the transcriptomes. However, as new KS units or veins develop in all of these leaves, our transcriptomes are useful for hypothesizing the regulatory genes involved in KS development. The samples also contained the shoot apical meristem (SAM), but as SAM is small compared with the leaves, its contribution to the transcriptomes may be small. In any event, our transcriptomes reveal a dynamic profile of transcriptional transition in parallel to anatomical transition in maize early leaf development during the first 72 h of seed germination.

Materials and Methods

A full description for *Materials and Methods* is provided in *SI Text*.

Plant Growth and Sample Collection. Seeds of *Zea mays* cv. White Crystal were imbibed. The embryonic leaves were taken every 6 h for 3 d for transcriptomic analysis. Details for plant growth conditions and anatomical studies are provided in *SI Text*, *Plant Growth Conditions and Sample Collection* and *SI Text*, *Anatomical Studies*, respectively.

RNA Sequencing and Data Processing. Total RNA was extracted, purified, quantified, and assessed for quality before Illumina sequencing. The library preparation steps and sequencing details are outlined in *SI Text*, *RNA Extraction and Sequencing*. Low quality reads were filtered based on quality scores. Read alignments to the B73 genome as well as gene expression level quantification were carried out following the “alternative protocol B” (28) as detailed in *SI Text*, *Data Processing and Analysis*.

Expression Profile Analyses. Genes were classified into coexpression modules based on the Pearson correlation coefficients of expression profiles between genes using hierarchical clustering (*SI Text*, *Expression Profile Correlation and Clustering*). We used the nonparametric method of Tarazona et al. (29) to identify DEGs between two samples (*SI Text*, *Identification of Differentially Expressed Genes*). Fisher’s exact tests were conducted to assess functional enrichment based on MapMan annotations (30).

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