

Genome-Wide Analysis of Alternative Splicing during Development and Drought Stress in Maize^{1[OPEN]}

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Alternative splicing plays a crucial role in plant development as well as stress responses. Although alternative splicing has been studied during development and in response to stress, the interplay between these two factors remains an open question. To assess the effects of drought stress on developmentally regulated splicing in maize (*Zea mays*), 94 RNA-seq libraries from ear, tassel, and leaf of the B73 public inbred line were constructed at four developmental stages under both well-watered and drought conditions. This analysis was supplemented with a publicly available series of 53 libraries from developing seed, embryo, and endosperm. More than 48,000 novel isoforms, often with stage- or condition-specific expression, were uncovered, suggesting that developmentally regulated alternative splicing occurs in thousands of genes. Drought induced large developmental splicing changes in leaf and ear but relatively few in tassel. Most developmental stage-specific splicing changes affected by drought were tissue dependent, whereas stage-independent changes frequently overlapped between leaf and ear. A linear relationship was found between gene expression changes in splicing factors and alternative splicing of other genes during development. Collectively, these results demonstrate that alternative splicing is strongly associated with tissue type, developmental stage, and stress condition.

After transcription, the majority of eukaryotic pre-mRNA is subjected to a series of posttranscriptional modifications, including the removal of introns to form a mature mRNA (Stamm et al., 2005). Although some introns are removed constitutively, many can be processed in a variety of alternative ways, including exon skipping, intron retention, alternative acceptor, alternative donor, and alternative position (change in both acceptor and donor positions; Lorković et al., 2000). These alternative splicing events form a crucial regulatory level and have the ability to alter an mRNA's stability, localization, and protein products. Alternative splicing events are controlled by a variety of cis-elements, including the presence of consensus splice sequences at the intron-exon border and intronic and exonic splicing enhancer sequences (Pertea et al., 2007). Trans-acting factors also exert

a strong effect on splicing and are mostly composed of Ser/Arg-rich proteins, which typically promote intron removal, and heterogenous nuclear ribonucleoproteins, which typically inhibit it (Erkelenz et al., 2013). A host of other indirect factors can affect splicing, including transcription rate, methylation status, and any cellular conditions that alter RNA secondary structure (Kornblihtt et al., 2004; Shepard and Hertel, 2008; Regulski et al., 2013). These different factors combine to determine the suite of isoforms that a gene produces in a given tissue, developmental stage, and environment.

Alternative splicing has been shown to play a role in a variety of different plant processes, including tissue identity (Staiger and Brown, 2013). Genes involved in processes ranging from auxin biosynthesis to iron transport have tissue-specific alternative splicing patterns that affect both protein localization and function (Kriechbaumer et al., 2012; Li et al., 2013). Tissue-specific splicing patterns can also alter a transcript's sensitivity to regulation by microRNAs, such as the maize (*Zea mays*) SPX family, which produces miR827-sensitive isoforms that predominate in developing seedlings, while insensitive isoforms dominate other tissues (Thatcher et al., 2014). Gene expression changes in splicing factors also play key roles in guiding tissue-specific developmental processes, including seed development, where the U2AF splicing factor ROUGH ENDOSPERM3 has been shown to be involved in mediating the interaction between the embryo and endosperm (Fouquet et al., 2011). The control of these

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alternative splicing differences among different tissues is thought to be the result of differential splicing factor expression and is likely also influenced by tissue-specific methylation patterns (Regulski et al., 2013). Alternative splicing has also been tied closely with nonsense-mediated decay (NMD) and has been shown to increase or decrease a transcript's sensitivity to this decay mechanism under a variety of conditions (Kalyna et al., 2012; Drechsel et al., 2013; Staiger and Brown, 2013).

Plants possess a wide array of regulatory mechanisms to deal with environmental stresses, which act at both the transcriptional and posttranscriptional levels. Alternative splicing has recently been implicated as playing a major role in these processes during stresses, including disease, heat, drought, and others (Deom et al., 1987; Iida et al., 2004; Jeong et al., 2011; Kakumanu et al., 2012; Chang et al., 2014; Mandadi and Scholthof, 2015). Gene expression changes in splicing factors are a major factor in determining stress-induced alternative splicing changes (Yan et al., 2012; Leviatan et al., 2013; Staiger and Brown, 2013). For example, Cui et al. (2014) recently showed that overexpression of the splicing factor *SAD1* conferred increased splicing precision and resulted in increased tolerance to salt stress in *Arabidopsis thaliana*. These and other findings highlight the importance of examining mRNA changes at both the transcriptional and posttranscriptional levels to obtain a more complete picture of both development and stress.

In order to assess the role of alternative splicing during drought and development, we constructed and analyzed 94 Illumina RNA-seq libraries from B73 developing leaf, ear, and tassel under well-watered and drought conditions. These data were supplemented with 53 publicly available maize B73 libraries from developing seed, endosperm, and embryo tissues (Chen et al., 2014). This analysis uncovered more than 48,000 novel isoforms of known genes and led to the identification of thousands of developmentally regulated splicing changes, many of which were affected by drought stress. These changes mostly occurred in leaf and ear, where drought caused a large increase in developmental splicing changes, while tassel was nearly unchanged by stress. Analysis of gene expression changes in genes associated with alternative splicing uncovered a linear relationship between the number of splicing factors with gene expression changes and the number of alternative splicing events in other genes

during development. Together, these results highlight the importance of alternative splicing in developing maize vegetative and reproductive tissues.

RESULTS AND DISCUSSION

Discovery of Novel Transcripts

To examine the regulation of alternative splicing during development under well-watered and drought field conditions, a series of 94 RNA-seq libraries was constructed and analyzed. This series included ear, tassel, and leaf tissue at four developmental stages (V12, V14, V18, and R1) under both well-watered and drought conditions, with four biological replicates each. After processing and genome matching (Kim et al., 2013), a total of over 1.3 billion genome-matched reads were utilized in downstream analysis (Table I). This data analysis was also supplemented with a publicly available time series of developing seed, endosperm, and embryo (Chen et al., 2014), which resulted in another 800 million genome-matched reads (Table I).

Genome-matched reads from all libraries were analyzed via Cufflinks (Trapnell et al., 2010) in order to assemble novel transcripts, which may have been missed previously due to tissue- or stage-specific expression. Novel transcripts were then filtered to remove low-abundance isoforms, which could be the result of sequencing processing intermediates or incorrect transcript assembly. Transcripts were required to have an average expression among four biological replicates of at least 1.3 fragments per kilobase per million reads (FPKM) in at least one stage and condition for ear, tassel, and leaf in order to optimize the tradeoff between false-positive and false-negative discoveries (Thatcher et al., 2014). Since many seed, endosperm, and embryo stages lacked biological replicates, the requirement for these tissues was an average of 1.3 FPKM in at least four biological replicates and/or consecutive developmental stages in at least one tissue. After abundance filtering, a total of 48,432 novel transcripts were identified (Supplemental Table S1).

Broad Tissue- and Stage-Specific Isoform Switches

In order to identify developmentally regulated changes in alternative splicing, Cuffdiff was used to quantify

Table I. Summary statistics for RNA-seq libraries

Description	Condition	Stages	Libraries	Total Reads	Genome Matched	Uniquely Mapped	Percentage Mapped
Ear	Watered	4	16	286,248,214	261,827,251	233,864,244	91
Tassel	Watered	4	16	215,381,271	205,082,097	180,285,669	95
Leaf	Watered	4	16	230,210,634	204,178,390	183,473,272	89
Ear	Drought	4	16	299,932,575	256,161,636	229,619,752	86
Tassel	Drought	4	14	207,741,601	197,232,548	173,040,626	95
Leaf	Drought	4	16	241,874,221	224,097,862	202,057,363	93
Seed	Watered	21	28	381,174,028	336,649,861	309,166,899	88
Endosperm	Watered	17	21	332,421,896	300,859,239	267,102,578	91
Embryo	Watered	15	16	217,398,766	197,532,915	189,422,320	91

transcript expression across all tissues, stages, and conditions (Trapnell et al., 2010). Each transcript's FPKM abundance was then converted into a percentage of total gene expression. In order to assess the relationship between the different tissues, stages, and conditions and their splicing profiles, we then performed principal component analysis (PCA). Isoform percentages from genes in all samples were utilized for PCA, with a relaxed cutoff of 1 FPKM total gene expression used for isoform quantification compared with isoform discovery. This initial analysis predominantly separated samples by tissue but lacked the resolution to easily separate stages and conditions (Supplemental Fig. S1). Two additional analyses were then carried out utilizing seed data (seed, endosperm, and embryo) and drought stress data (well-watered and drought-stressed ear, leaf, and tassel) separately. The analysis of seed data clustered samples by tissue type, with seed samples typically falling in between endosperm and embryo (Fig. 1A). Later stages of development of embryo and endosperm tended to cluster farther apart from each other than early stages, in keeping with their divergent developmental programming. These results are largely consistent with an earlier analysis of the same data at the gene expression level carried out by others, implying that splicing follows a similar pattern to gene expression in the developing maize kernel (Chen et al., 2014).

Samples included in the analysis of leaf, ear, and tassel data separate more clearly based on tissue type than those found in seed (Fig. 1B). Consistent with the distinct expression profiles of reproductive versus vegetative tissue, leaf clusters the farthest away from ear and tassel. Ear and tassel splicing profiles are highly similar at early developmental stages but diverge as development progresses, a pattern that fits well with the morphological similarities of these two tissues during early development compared with later stages (Cheng et al., 1983). Under drought conditions, tassel remains largely unchanged, with well-watered and drought-stressed samples overlaid nearly exactly on top of each other (Fig. 1B). This lack of response at the level of splicing is in keeping with the lack of strong phenotypic changes to tassel under drought (Herrero and Johnson, 1981). Ear growth is known to be strongly reduced under drought conditions (Boyer and Westgate, 2004), and large splicing differences based on drought treatment were observed (Fig. 1B). Leaf samples were taken from the middle of the developing leaf and underwent comparatively fewer morphological changes over normal development and also showed few developmental splicing differences. However, later stages of drought (V18 and R1) clustered apart from all other leaf samples, indicating an increased splicing response as drought progressed (Fig. 1B).

In order to examine developmental splicing changes in more detail, regression analysis was carried out on the relative isoform percentages of all genes above 1 FPKM across all developmental stages of a given tissue. In order to be considered to have a significant trend during development, an isoform's relative gene

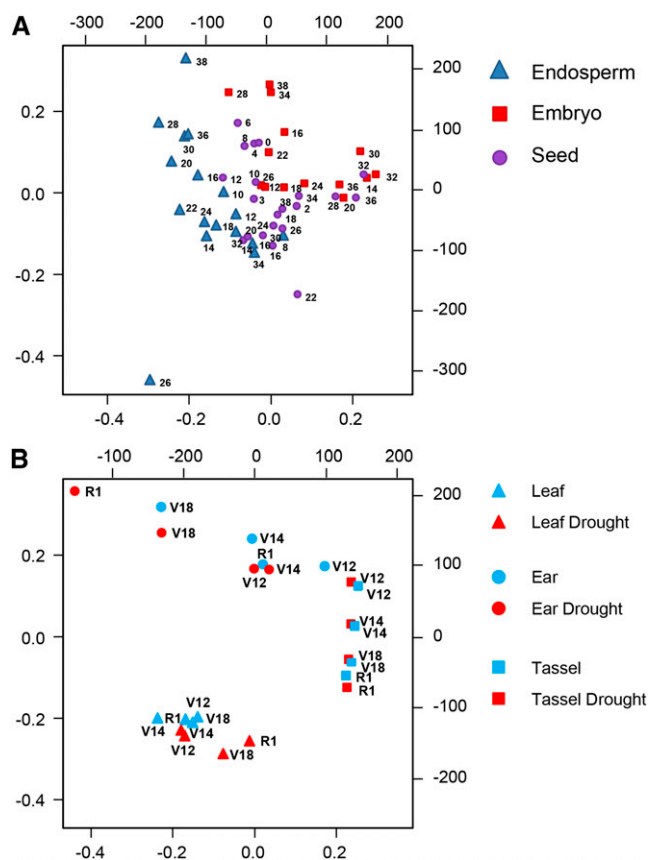


Figure 1. PCA of relative isoform percentages for all genes. A, Endosperm and embryo show clear separation, with seed falling in between. Stage numbers represent d after pollination (DAP). B, Leaf, ear, and tassel are clearly separated, with leaf possessing the most distinct isoform profile. Stages are shown from vegetative stage 12 (V12) through reproductive stage 1 (R1). Drought-stressed tissues are clearly separable for some stages of ear and leaf but indistinguishable for tassel.

representation was required to change by at least 30 percentage points (e.g. representing 10% of total gene expression at the first stage and 40% at the last) and its expression pattern was required to fit a linear or exponential model at $r^2 \geq 0.65$. The r^2 cutoff of 0.65 was chosen by running regression analysis on correctly ordered developmental series and comparing the number of trends found with the number found using the same analysis with randomized developmental order at r^2 cutoffs ranging from 0.1 to 0.95. At r^2 of 0.65, the average false-positive rate dropped below 5% (Supplemental Fig. S2, A and B). Potential overlap between tissues or conditions was determined by fitting expression values from one condition onto significant trend lines of another, with a significance cutoff of $r^2 \geq 0.3$. This relaxed cutoff was chosen to capture any potential overlap in order to reduce the chance of falsely assigning tissue specificity to a splicing change. Despite this lower cutoff, the average isoform change that overlapped between two tissues fit with an r^2 of 0.6. Isoform expression information for significantly regulated

genes in all tissues and conditions can be found in Supplemental Table S2.

Predominant Developmental Splicing Types Are Tissue Specific

Next, we categorized the differences between the isoform that lost the most relative gene representation and the isoform that gained the most relative gene representation over the course of development for each significant gene. This pairwise comparison revealed that roughly half of the developmental isoform changes are the result of alternative 5' start sites or modified 3' tails (Supplemental Table S3), with many isoform changes composed of multiple splicing and start site modifications (Table II). Alternative donor/acceptor changes represent around 40% of the total developmental splicing changes, with little variation between tissue types (Table II). Exon skipping, on the other hand, is comparatively rare among most tissue types (5% of all events) except in developing ears, where it represents more than 27% of all events under both well-watered and drought conditions. Exon addition is similarly rare in most tissues (6% of all events) but dominates developmental splicing in drought-stressed leaves, where it represents over 45% of all events. Intron retention/excision is the second most common difference in most tissues, but it is much rarer in developing ears and leaves. Overall, the tissue-specific nature of these different developmental splicing biases implies that splicing changes are likely controlled by changes in different splicing factors in different tissues.

NMD Sensitivity Is Affected by Isoform Switching during Development and Drought

Alternative splicing and transcription start sites have the potential to alter a transcript's sensitivity to NMD (Rayson et al., 2012). Although the rules governing

which transcripts are sent through the NMD pathway remain to be fully elucidated, recent studies suggest that three major factors in plants include 3' untranslated regions (UTRs) longer than 350 nucleotides, exons within 3' UTRs, and open reading frames greater than 35 amino acids within 5' UTRs (Kalyna et al., 2012). To assess the possibility that isoform switching interacts with the NMD pathway during development and drought, the isoform that lost the most relative gene representation was compared with the isoform that gained the most relative gene representation for all genes with significant isoform switching during drought or development. Overall, about half of all isoform switches were found to result in a change in the dominant isoform's likelihood of being targeted for NMD (Table III). This ratio is relatively constant across all tissues and conditions. Furthermore, no obvious bias was found toward a general decrease or general increase in NMD over the course of development or drought stress. Together, these findings suggest that NMD may play a major role in around half of all individual development- and stress-specific isoform-switching events, but neither development nor drought resulted in an overall increase or decrease in splicing-related NMD changes.

Seed, Embryo, and Endosperm Undergo Hundreds of Developmental Isoform Changes

Seed, embryo, and endosperm had 15 to 21 developmental stages, and trends were required to contain at least 10 of these stages. Seed, which contained a mixture of many different tissues, had the most significant isoform trends (687), followed by endosperm (483) and embryo (369). In total, 43% of the significant transcript changes from endosperm and 49% from embryo overlapped with those found in seed, indicating a good degree of reproducibility despite seed containing a mixture of different tissues (Fig. 2A). In contrast, only 100 of the 752 trends found in endosperm and embryo

Table II. *Isoform switch categories by tissue type and condition*

Category	Alternative Acceptor	Alternative Donor	Exon Addition	Exon Skipping	Intron Excision	Intron Retention	Alternative Start	Alternative Tail
Seed	65	51	36	30	81	62	309	337
Embryo	34	24	14	15	42	38	138	155
Endosperm	42	41	13	18	52	27	200	206
Leaf, watered	2	1	0	0	1	2	13	9
Leaf, drought	18	13	37	1	4	9	70	57
Ear, watered	24	16	10	31	14	16	116	101
Ear, drought	199	140	35	210	100	83	1,008	749
Tassel, watered	91	80	48	32	125	50	408	446
Tassel, drought	101	77	53	43	133	57	400	418
Leaf, drought stage independent	188	129	146	47	62	93	386	409
Ear, drought stage independent	169	105	80	54	62	78	323	351
Tassel, drought stage independent	1	2	0	0	5	3	1	7

Table III. NMD changes by tissue type and condition

Category	Same NMD	Higher NMD	Lower NMD	3' Exon Addition	3' Exon Loss	Long 3' Addition	Long 3' Loss	5' Upstream Open Reading Frame Addition	5' Upstream Open Reading Frame Loss
Seed	198	135	126	68	61	77	80	76	61
Embryo	84	84	40	52	22	56	20	43	37
Endosperm	111	87	93	35	54	57	39	47	55
Leaf, watered	11	3	4	1	3	2	3	1	1
Leaf, drought	44	17	24	14	6	4	11	11	21
Ear, watered	65	51	33	17	16	25	18	37	21
Ear, drought	591	362	225	66	143	230	102	248	142
Tassel, watered	249	165	156	77	75	103	106	94	78
Tassel, drought	233	155	172	82	81	97	115	89	90
Leaf, drought stage independent	304	205	190	111	96	114	110	100	93
Ear, drought stage independent	284	156	172	89	97	96	93	83	92
Tassel, drought stage independent	5	2	1	2	1	3	1	0	0

were shared by the two tissues, while 67 out of the 1,149 developmentally regulated transcripts were found in all three tissue types (Fig. 2A). The low degree of overlap between endosperm and embryo indicates a highly tissue-specific regulation of developmental isoform switching, especially when compared with the much higher overlap with seed. The overall larger amount of significant trends found only in seed may be the result of it containing multiple tissue types, but it could also be influenced by having the most stages present in our analysis (Table I). To assess this possibility, the same analysis was run utilizing only the final 15 stages of seed, which overlapped with embryo and endosperm. This smaller data set reduced the number of seed-only significant trends from 397 to 230, bringing it in line with endosperm and embryo.

In order to estimate the false-positive rate of significant trends for each tissue, the same regression analysis was rerun in all three tissues with stages placed out of the correct developmental order. For example, seed samples were placed in the order 21, 10, 20, and 9 DAP, etc. This disordered data set yielded dramatically reduced significant trends, with seed, endosperm, and embryo yielding 33, 14, and 11 significant trends, respectively. The large reduction in significant changes in an incorrectly ordered time series indicates that the findings in the correctly ordered set are of high confidence and likely have a false-positive rate of around 3% to 5%. This false-positive rate is also consistent with the 5% error rate that has been predicted when selecting an r^2 of 0.65 for significant trends in a regression analysis (Bryhn and Dimberg, 2011).

Although significant changes in splicing take place across all developmental stages, it is possible that certain stages may have higher or lower average rates of change. To assess this possibility, the SD of all significantly regulated isoforms within overlapping three-stage windows was calculated. Variability was seen in all tissues, with the most consistent three-stage windows having an average SD of 4.9 percentage points, while others had up to 7.7 percentage points. All three tissues showed a virtually identical pattern of average

isoform variability (Fig. 2B). Valleys representing periods of relative consistency around 3, 18, and 34 DAP and peaks with much higher rates of change around 8 and 28 DAP were present at similar levels in all three tissues (Fig. 2B). This highly consistent pattern of isoform variability is noteworthy given the relatively low level of overlap of individual transcripts (Fig. 2A) and indicates that shifts in isoforms may be controlled by one or a few master regulatory networks, such as changes in hormone levels. This is also consistent with an earlier dendrogram analysis of gene expression in the developing seed, which clustered stages into early, middle, and late development with transition points at 8 and 28 DAP (Chen et al., 2014). Taken together, these results indicate that gene expression and isoform switches are highly correlated in the developing maize seed and display similar periods of consistency and variability.

A small number of genes contain significant isoform switches in all three tissue types (Fig. 2A). This group is exemplified by GRMZM2G104658, a kinase with unknown function. GRMZM2G104658 is highly alternatively spliced and makes nine different known transcripts. Over the course of development, all three tissue types gradually reduced isoform 1 and increased isoform 7. This change resulted in a switch from a protein encoding only the kinase domain to one that also includes a small ligand-binding site that typically has regulatory function, which implies that GRMZM2G104658 may play a previously unknown role in the developing seed (Fig. 3A).

Although the majority of significant changes in isoforms were tissue specific, the functional classification of genes encoding them frequently overlapped. Several genes involved in hormone synthesis or response were significantly regulated, including *NAC TRANSCRIPTION FACTOR109* (*NACTF109*; GRMZM2G014653). *NACTF109* is known to produce five different transcripts, which mostly result in alterations of the protein's C-terminal domain. A novel transcript was identified in embryo, which also codes for a protein with an altered C terminus immediately following the

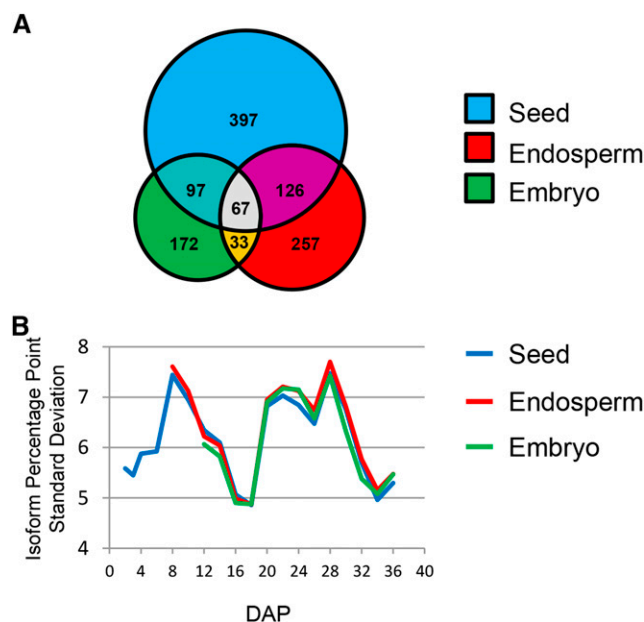


Figure 2. Alternative splicing changes over the course of seed, endosperm, and embryo development. A, Significant isoform changes over the course of development in seed, endosperm, and embryo. Transcripts are considered significant if their relative gene representation changed by at least 30 percentage points over the course of development and fit a linear or exponential regression at $r^2 \geq 0.65$. B, Average sd of all significantly regulated isoforms within overlapping three-stage windows showing that seed, endosperm, and embryo all have similar stage-specific isoform variation rates.

highly conserved NAC transcription factor domain (Fig. 3B). Over the course of development, embryo gradually switched from producing known isoform 3 to this novel isoform with an altered tail (Fig. 3B). *NACTF109*'s Arabidopsis homolog, *ANAC2*, regulates ABA biosynthesis and has been implicated in both development and stress regulation (Wu et al., 2009; Jensen et al., 2013; Garapati et al., 2015). The alternative splicing change in the maize *NACTF109* C-terminal region may result in altered protein localization or activity and may also affect its NMD targeting due to a change in 3' UTR length. This, in turn, could affect ABA levels in the developing embryo, where it is known to control dormancy and other processes (Rivin and Grudt, 1991).

A number of genes associated with disease responsiveness were also regulated over the course of development, predominantly in endosperm. This group is exemplified by GRMZM2G028568, a homolog of the Arabidopsis wall-associated kinase disease response factor *LEAF RUST10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-LIKE* (*LRK10L*). A novel maize *LRK10L* isoform with an alternative transcription start site and splicing pattern was identified during transcript discovery. This isoform lacks the extracellular galacturonan-binding wall-associated kinase (GUB WAK) domain and codes for a different downstream Cys-rich WAK domain (Fig. 3C). Over the

course of development, the novel isoform gradually declines and known isoform 1 comes to dominate *LRK10L* expression (Fig. 3C). The regulation of this and other disease resistance genes over the course of endosperm development may be the result of increasing pressure from pathogens as the maize kernel transitions through its grain-filling stage and increases its carbohydrate content, or it could point to previously unknown developmental roles for these genes (Myers et al., 2000).

Several genes associated with creating or reading epigenetic marks were found to be alternatively spliced over development in all three tissues. This group is represented by the methylation-sensitive *DDT-TRANSCRIPTION FACTOR6* (*DDT6*; GRMZM2G314661). Two novel isoforms of *DDT6* were identified, which differ by a minor change in a splice site acceptor and a small additional intron removal (Fig. 3D). The splice site acceptor modification results in the gain of a histone-interacting WHIM domain in novel isoform 2 compared with isoform 1, but the downstream intron removal causes the loss of a plant homeodomain fold involved in methylated histone H3 binding in this same isoform (Fig. 3D). Additionally, the second novel isoform has a dramatically increased 3' UTR with multiple exons, making it a likely target for NMD (Fig. 3D). Early in development of the seed, isoform 2 dominates *DDT6* expression, but the two isoforms gradually come to roughly equal expression levels by 21 DAP (Fig. 3D). Epigenetic marks have been associated previously with tissue identity (Luo et al., 2009), and here, we show that genes involved in epigenetic pathways are dynamically regulated at the level of alternative splicing as development progresses in the seed.

Ear, Tassel, and Leaf Have Thousands of Developmentally Regulated Isoform Changes under Well-Watered and Drought Conditions

Next, we examined significant developmental trends in leaf, ear, and tassel under both well-watered and drought conditions. Under well-watered conditions, a total of 1,204 isoforms had significant developmental regulation, while 3,064 were found during drought. Similar to the analysis conducted on seed samples, we determined the reliability of this data set by testing it with a disordered set: V18, V12, R1, and V14. This disordered data yielded only 40 and 22 significant changes under well-watered and drought conditions, respectively. The dramatic reduction in significant changes in a disordered time series again indicated that the findings in the correctly ordered set are likely to have a false-positive rate of around 2% to 4%.

In order to determine the overlap of developmentally regulated transcripts between different tissues, data from one condition were fitted onto significant trend lines generated from regression analysis of the other condition, with a relaxed criterion of $r^2 \geq 0.3$ (Fig. 4A). Analysis of tissue-level overlap showed that the vast

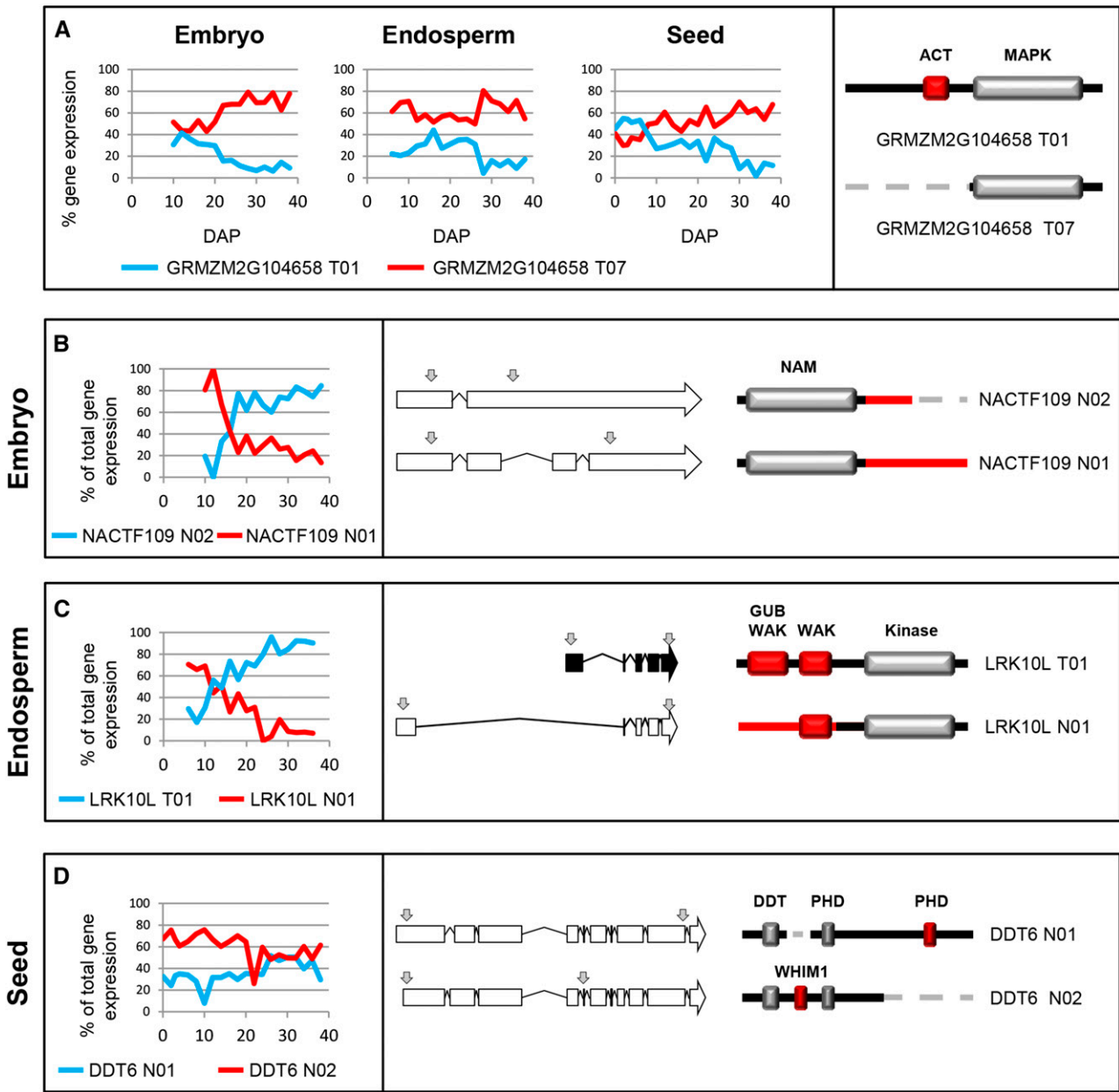


Figure 3. Examples of genes with developmentally regulated alternative splicing in embryo, seed, and endosperm. The two most abundant transcripts quantified by RNA-seq for each gene are shown. For transcript models, black denotes a known transcript while white is a novel transcript assembled by Cufflinks. Start and stop coordinates for the longest predicted proteins are marked by gray arrows. For protein models, conserved domains that are lost or gained are denoted in red while consistent domains are depicted in gray. A, Kinase GRMZM2G104658 switches between a protein with or without a small ligand-binding ACT domain in embryo, endosperm, and seed development. B, The abscisic acid (ABA) synthesis regulator *NACTF109* switches from producing a protein with a longer C-terminal tail to a shorter tail over embryo development. C, Disease resistance-related *LRK10L* switches from producing a protein without a GUB WAK domain to one with the GUB WAK over the course of endosperm development. D, Methylation-sensitive transcription factor *DDT6* exchanges an additional plant homeodomain (PHD) for a WHIM domain during seed development.

majority of developmental changes were tissue specific (Fig. 4A). Under well-watered conditions, very little overlap was seen between ear and tassel (19 transcripts), and leaf did not share any developmental regulation with either other tissue. Under drought, ear

increased its overlap with tassel (34 transcripts) and gained a low level of overlap with leaf (eight transcripts). Overall, this low level of overlap indicated a high degree of tissue specificity in developmental isoform switches.

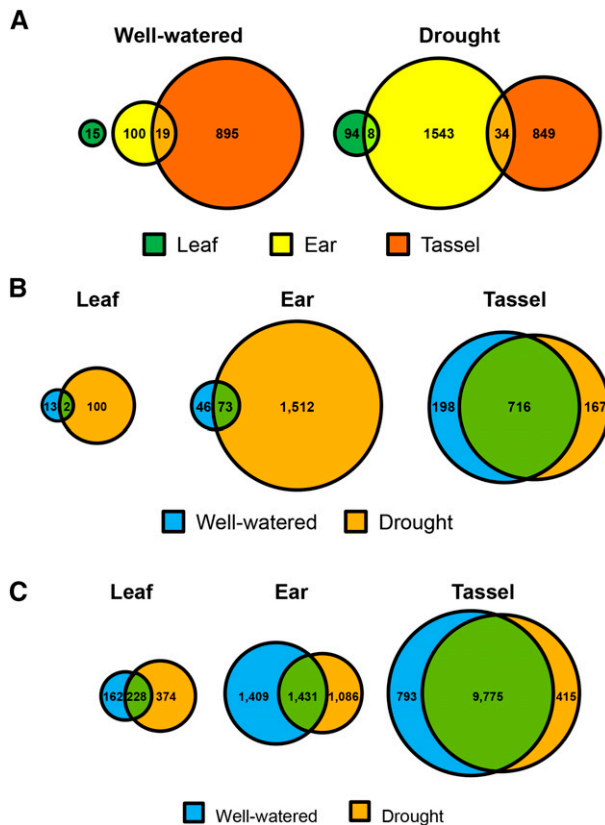


Figure 4. Significant isoform and gene expression changes over the course of development and drought in leaf, ear, and tassel. Changes in transcripts were considered significant if their relative gene representation changed by at least 30 percentage points over the course of development and fit a linear or exponential regression at $r^2 \geq 0.65$. A, Overlap between developmental isoform switches in different tissues. B, Overlap between developmental isoform switches under well-watered and drought conditions. C, Overlap of developmental gene expression changes between well-watered and drought conditions in leaf, ear, and tassel.

In keeping with the earlier PCA results (Fig. 1), leaf underwent the smallest total number of isoform switches over the course of development, with only 15 isoforms showing a significant change under well-watered conditions. Only two of these changes were also found in drought at $r^2 \geq 0.3$, suggesting that drought causes a large deviation from normal developmental programming (Fig. 4B). This difference can be clearly seen in *CORONATINE INSENSITIVE1* (*COI1*; GRMZM2G353209), a jasmonate-responsive component of a ubiquitination complex involved in defense and drought responses (Xie et al., 1998; Harb et al., 2010). Transcript discovery revealed a novel isoform of *COI1* that codes for a similar protein that retains a crucial F-box, shown to be crucial for ubiquitination target selection in Arabidopsis, but lacks a Leu-rich repeat region (Fig. 5A; Xu et al., 2002). Under well-watered conditions, *COI1* gradually changes from producing the known full-length isoform to production of the novel isoform (Fig. 5A). Under drought

conditions, *COI1* continues to produce the known isoform containing the Leu-rich repeat region, potentially resulting in increased jasmonate sensitivity in older leaves under drought compared with well-watered conditions.

Substantially more genes with isoform switching over the course of development were found when leaves were stressed with drought than under well-watered conditions (Fig. 4B). This group included several genes associated with stress response, such as *HEAT SHOCK PROTEIN93-V* (*HSP93-V*; GRMZM2G009443), a chloroplast-associated heat shock protein. A novel isoform of *HSP93-V* that lacks both Clp protein-binding sites was identified (Fig. 5B). Under well-watered conditions, this truncated isoform predominates, but drought causes a gradual increase in the full-length known transcript containing these Clp domains (Fig. 5B). Clp domains are crucial for the function of this class of heat shock protein, and the switch to an isoform that codes for them under drought stress conditions could function to increase protein degradation or prevent protein aggregation (Wang et al., 2004).

Ear showed significantly more developmental regulation of isoform switching under well-watered conditions compared with leaf (119 isoforms), and the majority of this regulation (73 isoforms) was consistent despite drought conditions. Still, large deviations from normal developmental changes were seen in some genes, such as *MONODEHYDROASCORBATE REDUCTASE4* (*MDAR4*; GRMZM2G320307). *MDAR4* is a membrane-bound monodehydroascorbate reductase that functions in the ascorbate-glutathione cycle to remove toxic hydrogen peroxide, but it also has been implicated in seed storage and postgermination growth in Arabidopsis (Chew et al., 2003; Eastmond, 2007). Under well-watered conditions, known isoform 1 slowly reduces over the course of development, while a second known isoform with a longer C-terminal tail steadily increases. This isoform also has a much shorter 3' UTR, which could potentially alter the transcript's sensitivity to NMD (Kalyna et al., 2012; Fig. 5C). Under drought conditions, isoform 1 continues to dominate the gene expression from V12 through R1. These changes in *MDAR4* may be a response to stress-induced increases in hydrogen peroxide but also could affect seed storage as development progresses to later stages.

During drought, ear showed substantially more developmental regulation of isoform switching (1,512 isoforms; Fig. 4B). In keeping with this large increase in alternative splicing during drought, several splicing factors were identified as drought regulated, such as the salicylic acid-responsive *RNA-BINDING PROTEIN-DEFENSE RELATED1* (*BRN1*; GRMZM2G005459). *BRN1* was found to produce a novel isoform that codes for a protein with two, rather than three, RNA-binding domains (Fig. 5D). Under well-watered conditions, this slightly truncated isoform gradually came to dominate *BRN1*'s total gene

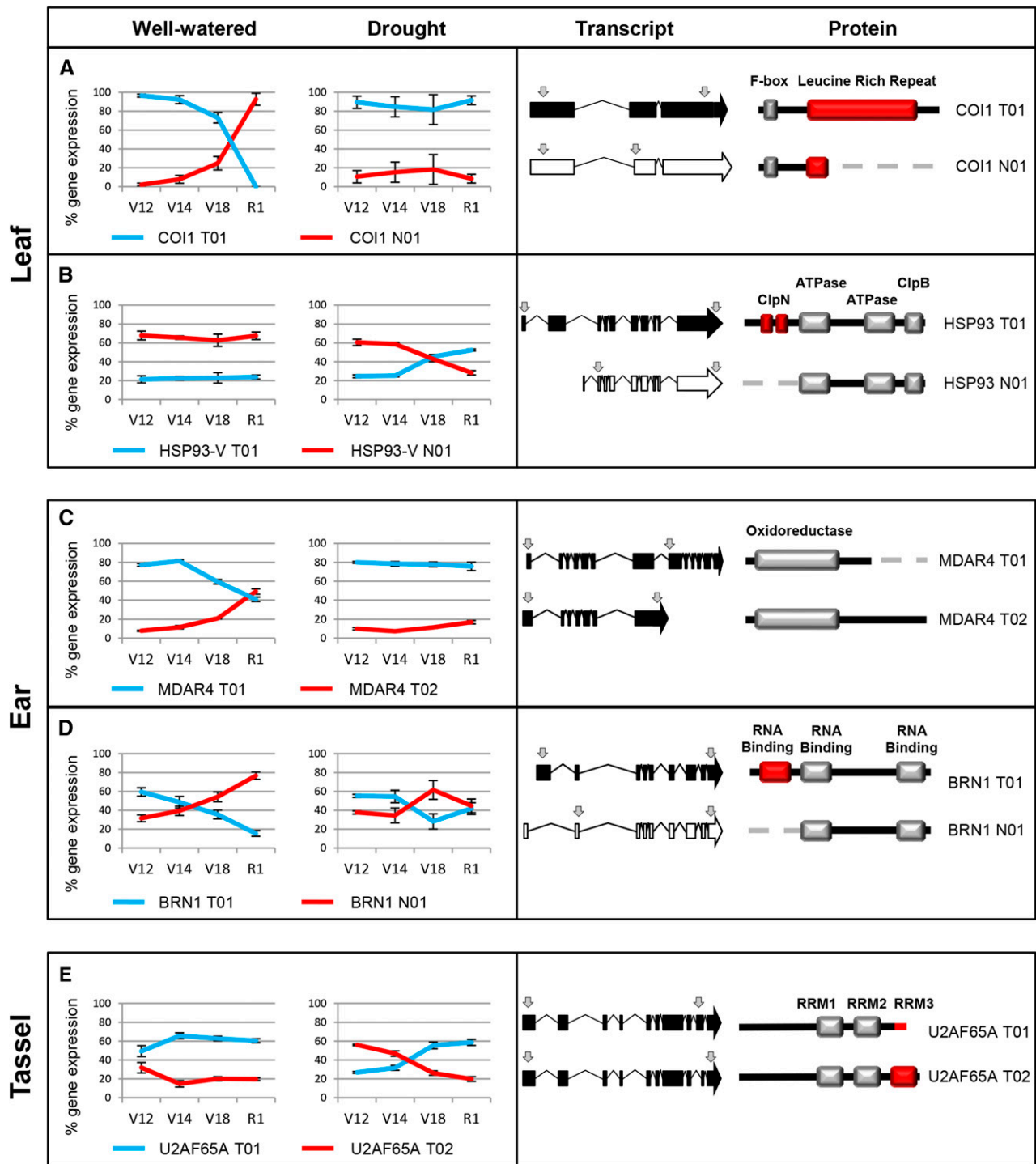


Figure 5. Examples of genes with developmentally regulated alternative splicing under well-watered and drought conditions in leaf, ear, and tassel. The two most abundant transcripts quantified by RNA-seq for each gene are shown. Error bars represent \pm SE from four biological replicates. For transcript models, black denotes a known transcript and white is a novel transcript assembled by Cufflinks. Start and stop coordinates for the longest predicted proteins are marked by gray arrows. For protein models, conserved domains that are lost or gained are denoted in red while consistent domains are depicted in gray. A, The splicing pattern of the jasmonate-responsive ubiquitination component *COI1* is changed during development in leaf only under well-watered conditions. B, The heat shock protein *HSP93-V* undergoes alternative splicing changes in leaf only under drought. C, *MDAR4* undergoes alternative splicing changes in ear under well-watered conditions. D, The splicing factor *BRN1* undergoes alternative splicing changes in ear only under well-watered conditions. E, The splicing factor *U2AF65A* undergoes alternative splicing changes in tassel under drought.

expression, potentially significantly affecting the splicing of many other genes (Fig. 5D). The regulation of this defense-related splicing factor in drought-stressed ears may represent a splicing-related response to increased disease pressure under more stressful conditions (Qi et al., 2010).

Tassel had the most developmental regulation under well-watered conditions (914 isoforms), and almost 80% (716 isoforms) of these maintained a similar pattern under drought conditions. This high degree of similarity in developmental splicing under well-watered and drought conditions was also in keeping with earlier PCA results (Fig. 1). Despite this, some genes did show significant differences under drought conditions. Splicing factor U2 small nuclear ribonucleoprotein auxiliary factor (*U2AF65A*; GRMZM5G813627) is known to produce a number of different functional isoforms, with alteration in its three RNA recognition motifs (RRMs). Under well-watered conditions, isoform 1, which contains RRM1 and 2, is always the dominant isoform. Under drought conditions, however, isoform 2 (which contains all three RRMs) dominates expression early on and gradually diminishes until, at R1, *U2AF65A*'s splicing pattern under drought becomes nearly identical to well-watered conditions (Fig. 5E). Another *U2AF* splicing factor, *ROUGH ENDOSPERM3*, has been implicated in seed development (Fouquet et al., 2011), while GRMZM5G813627 may instead play a role in tassel.

Developmentally Consistent Drought-Induced Isoform Changes

Next, we analyzed drought-induced isoform changes that occurred in a developmental stage-independent manner. Isoform percentages for genes with greater than 1 FPKM expression in at least three out of four developmental stages under both well-watered and drought conditions were compared with each other. Isoform abundances with an average of at least a 30 percentage point difference between well-watered and drought conditions were then compared between conditions utilizing Student's *t* test (critical value 0.999). Thousands of consistent changes were seen, including 1,060 in leaf, 932 in ear, but only 11 in tassel (Fig. 6A; Supplemental Table S4). Tassel's low number of consistent isoform changes under drought was similar to its small changes in developmentally controlled isoform switching under drought (Fig. 6B). Stage-independent drought isoform changes in leaf and ear, on the other hand, were significantly different. Leaf had the smallest number of developmental changes under both drought and well-watered conditions but showed the most stage-independent isoform changes during drought (Figs. 4B and 6A). In contrast to developmentally controlled drought responses, where ear and leaf had very little overlap, developmental stage-independent changes were

highly similar between these tissues (Figs. 4A and 6A). These findings indicate that stage-specific drought alternative splicing tends to be highly tissue specific, while stage-independent drought changes occur in a number of tissues simultaneously.

A comparison of the types of alternative splicing present in leaf and ear under drought conditions revealed a fairly similar distribution of categories to that seen in developmental splicing (Tables II and III). Alternative donor/acceptor dominated the alternative splicing seen in leaf and ear to a greater degree. Unlike developmental splicing changes, drought-induced splicing showed no clear bias toward skipping or

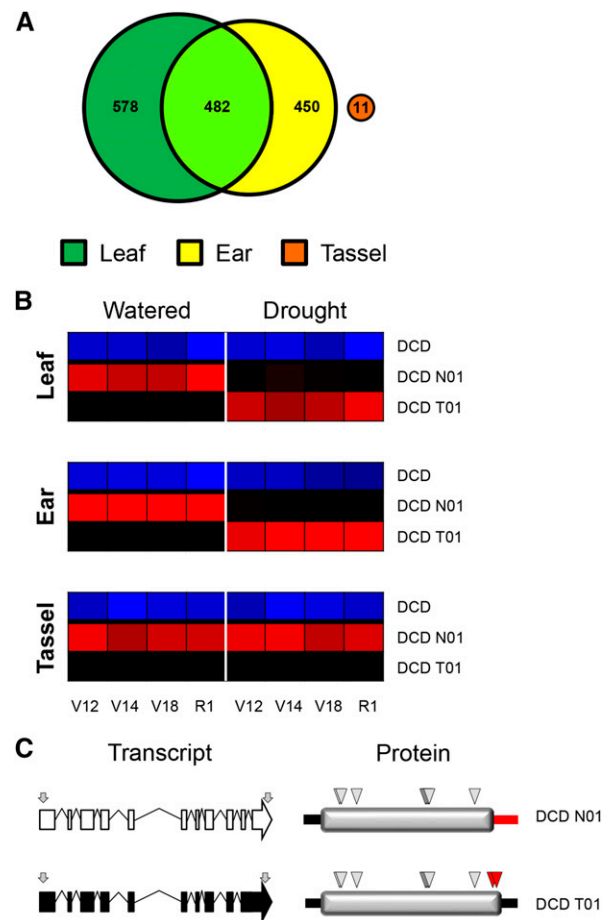


Figure 6. Significant isoform switches between well-watered and drought conditions that are consistent across developmental stages. **A**, Tissue overlap of significant drought-induced alternative splicing changes that were consistent across at least three out of four developmental stages. **B**, *DCD* (GRMZM2G151440) gene expression is unaffected by drought but undergoes significant changes in alternative splicing in leaf and ear. Gene expression comparison between samples is shown in blue. Relative gene representation of the two isoforms within a sample is shown in red. **C**, *DCD* splicing changes affect highly conserved amino acid residues (arrowheads) within the catalytic domain (gray boxes). Amino acid residues and protein regions that are altered during the isoform switch are denoted in red. Start and stop coordinates for the longest predicted protein are marked by gray arrows.

addition. Overall, stage-independent drought splicing categories were much more similar in leaf and ear than changes seen during their development, which was consistent with the much higher overlap of individual transcripts in the stage-independent splicing set (Fig. 6A). Tassel had very few stage-independent drought splicing events, but eight out of 11 of these involved a change in intron retention/excision, which was also the predominant category seen during tassel development under well-watered and drought conditions (Tables II and III).

Many stage-independent changes looked virtually identical in leaf and ear but were completely absent in tassel, a trend exemplified by *D-CYSTEINE DESULFHYDRASE* (*DCD*; GRMZM2G151440). Under drought conditions, *DCD* undergoes a consistent and dramatic switch from a novel slightly truncated isoform to its known full-length isoform in both leaf and ear, but it is unchanged in tassel (Fig. 6B). The known isoform, which is induced under drought, contains the full substrate-binding pocket, compared with the novel isoform, which dominates under well-watered conditions (Fig. 6C). Interestingly, *DCD*'s Arabidopsis homolog has been shown to be induced during drought stress and is thought to confer drought tolerance through increased production of hydrogen sulfide (Jin et al., 2011). Maize *DCD* is not regulated at the gene expression level in our data but instead undergoes a splicing change that increases the ratio of fully active protein, potentially causing increased hydrogen sulfide production through alternative splicing instead of gene expression (Fig. 6, B and C).

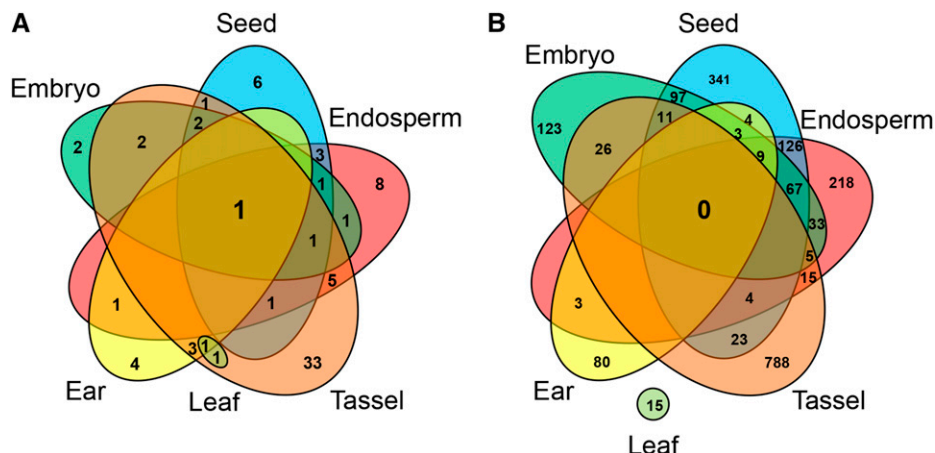
Changes in Splicing Correlate with Gene Expression Changes in Splicing Factors

Next, we analyzed gene expression over the course of development. Genes were considered significantly regulated if they had more than a 3-fold change and fit a linear or exponential regression model at $r^2 \geq 0.65$.

Although the total number of genes with significant expression changes was greater than those with isoform changes, the pattern of both tissue- and condition-specific regulation was largely similar to that seen in the alternative splicing data (Fig. 4). One exception to this similar pattern was drought-stressed ears, which underwent a large induction in developmental isoform switching but only a modest change in developmental gene expression changes during drought (Fig. 4, B and C). Additionally, although seed underwent the largest number of developmental isoform switches, endosperm underwent the largest number of developmental gene expression changes (Fig. 2A; Supplemental Fig. S3).

A more focused analysis was then carried out on the expression change of genes associated with alternative splicing regulation based on Gene Ontology (GO). A total of 293 maize genes that had GO terms associated with splicing or had orthologs in Arabidopsis or rice (*Oryza sativa*) associated with splicing were examined. A total of 77 known or putative splicing factors were found to be regulated in at least one tissue and condition, with 31% occurring in more than one tissue (Fig. 7A; Supplemental Table S5). A similar comparison of splicing changes across all six tissues yielded even more tissue specificity, with only 10% of all splicing changes occurring in more than one tissue (Fig. 7B). The difference in specificity of splicing factor gene expression changes compared with splicing changes likely arises due to the influence of other factors on splicing, such as transcription rate or methylation status (Kornblihtt et al., 2004; Shepard and Hertel, 2008; Regulski et al., 2013). It has been shown previously that splicing factors are frequently alternatively spliced, often resulting in increased or decreased NMD sensitivity (Lareau and Brenner, 2015). In total, we identified 77 splicing factors with gene expression change (Supplemental Table S5) and 46 splicing factors that are alternatively spliced. However, only six of these potential splicing factors were subject to both layers of regulation during

Figure 7. Significant expression changes in genes associated with splicing and significant alternative splicing changes in all genes. A, Tissue overlap of genes with GO terms associated with splicing whose expression changes by more than 3-fold and fits a linear or exponential regression line at $r^2 \geq 0.65$. B, Tissue overlap of splicing isoforms whose relative gene representation changes by more than 30 percentage points and fits a linear or exponential regression line at $r^2 \geq 0.65$.



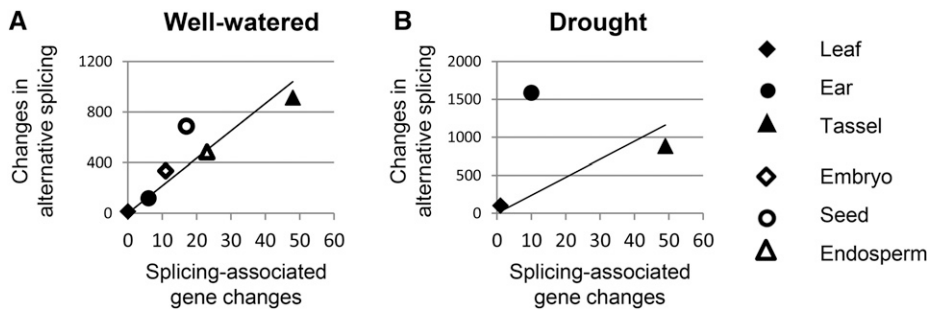


Figure 8. Number of genes with significant alternative splicing changes versus the number of splicing-related genes with expression changes. A, Well-watered conditions show a linear relationship between the number of genes associated with splicing that are under expression regulation and the number of alternatively spliced genes. B, Under drought, no clear relationship is seen between splicing gene regulation and alternative splicing changes.

development, none of which showed signs of gene expression regulation via NMD sensitivity changes (Supplemental Tables S2 and S5).

When comparing across all tissues under well-watered conditions, the number of splicing-regulating genes with developmental expression changes has a linear relationship with the number of genes that underwent alternative splicing changes over development (Fig. 8A). On average, for every splicing factor with significant gene expression change, 21 genes have significant splicing changes. This trend held despite the use of disparate tissue types, with time point numbers ranging from four to 21. Under drought stress, the trend no longer held across tissues (Fig. 8B). The loss of the linear relationship of splicing factor regulation with alternative splicing under drought may be due to the effects of transcription rate or osmotic stresses on RNA secondary structure, both of which could potentially alter splicing independent of changes in the expression level of splicing factors (Shepard and Hertel, 2008). Additionally, the massive increase in developmental splicing changes in drought-stressed ears may be due to an increase in the number of putative splicing factors that are alternatively spliced from three to 24.

Intriguingly, the expression level of splicing factor *PRP18* (GRMZM2G102711) seems to correlate well with drought-responsive alternative splicing. Under well-watered conditions, this gene has only minor regulation over development. However, ear and leaf, which show dramatic increases in splicing under drought conditions, show large increases in expression for this gene under drought conditions (Fig. 9).

On the other hand, no expression change for this gene was seen in tassel, which had few drought-induced developmental splicing changes (Figs. 8 and 9). In *Arabidopsis*, overexpression of the salt stress-regulated splicing factor *SAD1* was shown to increase splicing precision and confer salt tolerance (Cui et al., 2014). *PRP18*, therefore, may play a similar role in drought stress response and warrants further investigation.

CONCLUSION

Deep sequencing of maize leaf, ear, and tassel tissues over development and during drought stress as well as inclusion of public data from developing maize seed, endosperm, and embryo tissues enabled the identification of thousands of developmental and stress-related splicing changes. Ninety percent of these splicing changes were specific to a single tissue, indicating a tight control of developmental alternative splicing changes. Drought was found to cause massive shifts in developmental splicing changes in leaf and ear but only minor changes in tassel. Dozens of known or putative splicing factors were found to be regulated at the gene expression level during development, and a linear relationship was found between expression changes in splicing factors and splicing changes in other genes. Taken together, these findings highlight the important role that alternative splicing plays in tissue-specific development and response to drought stress in maize.

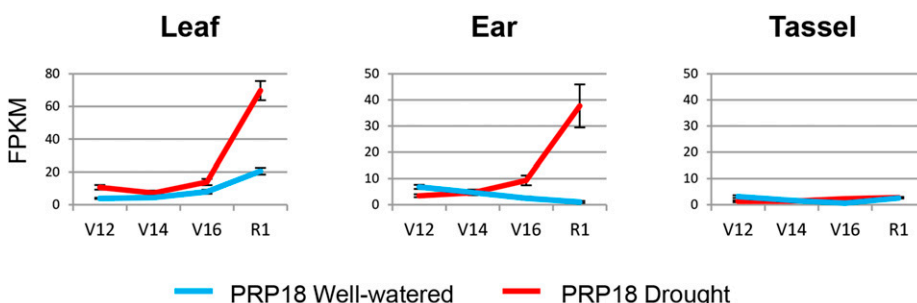


Figure 9. Splicing factor *PRP18*'s expression change over the course of development in leaf, ear, and tassel. Gene expression for *PRP18* (GRMZM2G102711) increases over development under drought stress in both leaf and ear but is unchanged in tassel. Data were generated from RNA-seq quantification. Error bars represent SE from four biological replicates.

MATERIALS AND METHODS

Plant Growth and Harvesting

B73 maize (*Zea mays*) plants were grown in a field in Woodland, California, in 2012. Plots were designed utilizing a randomized complete block design with four biological replicates. Drought stress was applied by withholding water after stage V8, while well-watered plants received constant irrigation to achieve maximum yields. Tissues were harvested 11, 18, 27, and 32 d later from both well-watered and drought-stressed plants. Time points corresponded to stages V12, V14, V18, and R1 for well-watered plants, with minor stage delays seen in drought-stressed plants. All tissues were harvested from the same plants. Leaf tissue was cut from the middle section of the top collared leaf from three different plants in each replicate group. Ears were selected based on size at each time point: approximately 0.5 cm, 0.8 to 1.5 cm, 2 to 4 cm, and 3 to 8 cm. The first time point was composed of 32 ears, the second 16 ears, and the third and fourth three to four ears. Whole tassels were dissected from whorls during the first two time points. Tassel emergence appeared at the third time point and pollen shedding at the last. After this point, 10 cm of the main spike was sampled. All tissues were immediately frozen in liquid nitrogen and then utilized for mRNA sequencing library preparation.

mRNA Sequencing Library Preparation and Sequencing

Total RNA was isolated from frozen maize tissues with the Qiagen RNeasy Kit for total RNA isolation. Libraries from total RNA were then prepared using the TruSeq mRNA-Seq Kit and protocol from Illumina and sequenced on the Illumina HiSeq 2500 system with Illumina TruSeq SBS version 3 reagents. On average, 18 million reads were generated from each library (Table I). Resulting sequences were trimmed based on quality scores (Phred score ≥ 13) and mapped to the maize B73 reference genome sequence V2 and maize working gene set V5a with TopHat2 version 2.0.14 (Kim et al., 2013) using several modifications from default parameters: maximum intron size, 100,000; minimum intron size, 20; and up to two mismatches allowed. Reads that aligned to multiple locations were assigned heuristically based on the abundance of surrounding regions (Kim et al., 2013). Libraries with less than 5,000,000 genome-matched reads (one biological replicate of well-watered R1 tassel and one biological replicate of drought-stressed R1 tassel) were excluded from later downstream analysis.

Computational Prediction of Novel Isoforms

Genome-matched reads from each library then were assembled with Cufflinks version 2.1.1 (Trapnell et al., 2010) using several modifications from default parameters: maximum intron size, 100,000; and minimum intron size, 20. Cuffmerge version 2.1.1 (Roberts et al., 2011) was then used to merge individual transcript assemblies into a single transcript set. The annotation of novel junctions required at least 10 reads spanning them and any new transcripts needed to represent at least 10% of the total gene abundance in at least one library. Known and novel transcripts were quantified in each tissue and genotype with Cuffnorm version 2.1.1 (Roberts et al., 2011) using default parameters. Novel transcripts with expression of less than 1.3 FPKM in all tissues and stages were filtered out.

Analysis of Developmental Isoform Changes

Cuffdiff version 2.1.1 (Roberts et al., 2011) was used to quantify transcript expression across all tissues, developmental stages, and conditions, which were then converted to relative percentage of total gene expression. Regression analysis was then applied to individual transcripts to look for linear or exponential developmental trends, with at least a 30 percentage point change and $r^2 \geq 0.65$ required to be considered significant.

The data set supporting the results of this article is available in the National Center for Biotechnology Information Gene Expression Omnibus repository (GSE71723).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. PCA analysis of relative isoform percentages for all tissues, stages, and conditions.

Supplemental Figure S2. Significant gene expression trends over the course of development.

Supplemental Figure S3. Number of isoform switches during development at various r^2 cutoffs.

Supplemental Table S1. Transcripts discovered during Cufflinks transcriptome assembly.

Supplemental Table S2. Relative isoform percentages of total gene expression.

Supplemental Table S3. Splicing changes versus alternative start site/tail.

Supplemental Table S4. Genes with consistent isoform switches under drought.

Supplemental Table S5. Significant gene expression changes during development.

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