Digital Gene Expression Signatures for Maize Development

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Genome-wide expression signatures detect specific perturbations in developmental programs and contribute to functional resolution of key regulatory networks. In maize (Zea mays) inflorescences, mutations in the RAMOSA (RA) genes affect the determinacy of axillary meristems and thus alter branching patterns, an important agronomic trait. In this work, we developed and tested a framework for analysis of tag-based, digital gene expression profiles using Illumina’s high-throughput sequencing technology and the newly assembled B73 maize reference genome. We also used a mutation in the RA3 gene to identify putative expression signatures specific to stem cell fate in axillary meristem determinacy. The RA3 gene encodes a trehalose-6-phosphate phosphatase and may act at the interface between developmental and metabolic processes. Deep sequencing of digital gene expression libraries, representing three biological replicate ear samples from wild-type and m3 plants, generated 27 million 20- to 21-nucleotide reads with frequencies spanning 4 orders of magnitude. Unique sequence tags were anchored to 3’-ends of individual transcripts by DpnII and NlaIII digests, which were multiplexed during sequencing. We mapped 86% of nonredundant signature tags to the maize genome, which associated with 37,117 gene models and unannotated regions of expression. In total, 66% of genes were detected by at least nine reads in immature maize ears. We used comparative genomics and tested a framework for analysis of tag-based, digital gene expression profiles using Illumina’s high-throughput sequencing technology and the newly assembled B73 maize reference genome. We also used a mutation in the RA3 gene to identify putative expression signatures specific to stem cell fate in axillary meristem determinacy. The RA3 gene encodes a trehalose-6-phosphate phosphatase and may act at the interface between developmental and metabolic processes. Deep sequencing of digital gene expression libraries, representing three biological replicate ear samples from wild-type and m3 plants, generated 27 million 20- to 21-nucleotide reads with frequencies spanning 4 orders of magnitude. Unique sequence tags were anchored to 3’-ends of individual transcripts by DpnII and NlaIII digests, which were multiplexed during sequencing. We mapped 86% of nonredundant signature tags to the maize genome, which associated with 37,117 gene models and unannotated regions of expression. In total, 66% of genes were detected by at least nine reads in immature maize ears. We used comparative genomics to leverage existing information from Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa) in functional analyses of differentially expressed maize genes. Results from this study provide a basis for the analysis of short-read expression data in maize and resolved specific expression signatures that will help define mechanisms of action for the RA3 gene.
wide tiling arrays provide resolution up to the single nucleotide level and have been utilized to identify transcript variants (such as alternatively spliced transcripts), single-feature polymorphisms, and epigenetic marks (for review, see Gregory et al., 2008). Although microarray technologies continue to evolve, the recent emergence of deep-sequencing platforms has motivated the current digital age of functional genomics (Blow, 2009; Lister et al., 2009, Metzker, 2010). Next-generation technologies, such as those developed by Illumina (previously Solexa), 454 Life Sciences (Roche), and Applied Biosystems (ABI), can generate tens of thousands (Roche-454) to tens of millions (Illumina and ABI) of sequence reads, in parallel, with exceptional reproducibility (Li et al., 2008; Marioni et al., 2008; Simon et al., 2009). Adapting these technologies to genome-wide expression studies circumvents the inherent limitations of hybridization-based methods. For example, sequence-based methods require no prior knowledge of sequence and/or transcript composition and thus provide a potentially unbiased view of the transcriptome, which is not limited to fully sequenced genomes (Blencowe et al., 2009; Simon et al., 2009). In addition, sequencing technologies enable the resolution of transcript variants and novel mRNAs (Sultan et al., 2008; Marioni et al., 2008), minimize biases due to cross-hybridization (Tang et al., 2009), and provide quantitative measures of transcript abundance based on read count over a wide dynamic range (’t Hoen et al., 2008; Mortazavi et al., 2008; Sultan et al., 2008; Morrissy et al., 2009; Babbitt et al., 2010).

A number of studies have used next-generation sequencing technologies for genome-scale expression analyses in higher eukaryotes. Such approaches include whole-transcript sequencing and assembly (RNA-seq) using the long-read, 454 platform (Emrich et al., 2007a; Weber et al., 2007) and the massively parallel Illumina (Mortazavi et al., 2008; Wang et al., 2008, 2009) and ABI SOLiD (Tang et al., 2009) systems. While Illumina and ABI achieve a much greater depth of sequencing, read lengths are significantly shorter (typically 36–75 bases) compared with 454 (up to 500 bases). Alternatively, tag-based approaches target 3’-ends of transcripts to generate short (15–21 base) signature sequences from individual mRNAs (Harbers and Carninci, 2005). Early tag-based sequencing using serial analysis of gene expression (Velculescu et al., 1995) yielded relatively low read depth and required laborious cloning steps. More recently, Illumina’s Digital Gene Expression (DGE) platform, upgraded from the previous massively parallel signature sequencing (MPSS) technology (Brenner et al., 2000; Jongeneel et al., 2003; Meyers et al., 2004), can generate, at its current capacity, 90 to 100 million reads per run of an eight-lane flow cell using the Genome Analyzer 2x (GA2x) system (www.illumina.com). Although whole-transcriptome sequencing methods provide information on alternative splicing (Pan et al., 2008; Sultan et al., 2008) and novel expression patterns from intergenic regions (Lister et al., 2008), the nonredundant nature of tag-based profiles would, in theory, allow for greater depth of sequencing per transcript. DGE produces a specific 3’ signature for each mRNA, thereby reducing library saturation from abundant transcripts and enhancing the capacity for rare transcript detection (’t Hoen et al., 2008; Asmann et al., 2009; Morrisey et al., 2009; Babbitt et al., 2010). Likewise, increased read counts per unique transcript would enhance power for statistical analyses in comparing quantitative expression profiles among samples. In addition, DGE data files can be collapsed to a smaller number of unique sequences, thus allowing for less storage requirements, more efficient mapping without the need for high-performance computing, and thus less bioinformatic support. Furthermore, the DGE protocol is strand specific and requires up to 10 times less starting RNA than current whole-transcript, RNA-seq approaches, which is a key advantage when tissue is limiting.

Until recently, an unsequenced genome and lack of adequate gene models and annotations have limited large-scale transcriptome analyses in maize. The maize genome is highly complex, having undergone two successive rounds of duplication (Messing and Dooner, 2006; Wei et al., 2007). Sequencing of the B73 maize reference genome revealed that approximately 81% of the genome could be assigned to homoeologous regions (Schnable et al., 2009). In addition, tandemly duplicated gene families occur frequently throughout the genome (Messing and Dooner, 2006; Schnable et al., 2009), and near-identical, paralogous genes (98% or greater identity) are often coexpressed (Emrich et al., 2007a). Such complications have recently been addressed by using sequence-based transcript profiling methods to identify novel genes (Emrich et al., 2007a), resolve the expression of family members and near-identical paralogs (Eveland et al., 2008), and quantify allelic variants (Barbazuk et al., 2007; Guo et al., 2008) in maize.

The shift to functional genomics studies in maize will be dependent on standardized methods for the analysis and assessment of the various sequencing methods with regard to specificity, mapability, depth of coverage, and cost. Despite rapid advances and extensive applications of next-generation sequencing technologies, methods for data analysis have not been well established. In this work, we evaluated the performance of short-read, DGE profiling in cataloging of gene-specific signatures at a particular stage of maize inflorescence development. We present a framework for genome-wide analysis of tag-based expression data in maize and describe a comprehensive pipeline for mapping short sequence reads, accessing gene information from Ensembl (Flicek et al., 2010), and quantifying differences in transcript abundance based on read counts using an open-source statistical package. We also show that analysis of tags mapping independently of known gene models can be used to identify unannotated transcripts, a clear advantage over microarrays. The analyses described here can also be adapted to RNA-seq data sets.

We also tested the effect of perturbing a key developmental pathway in inflorescence architecture using
a mutant in the RAMOSA3 (RA3) gene. RA3 encodes a trehalose-6-phosphate phosphatase (TPP) and functions in regulating the determinacy of axillary meristems (Satoh-Nagasawa et al., 2006). Although RA3 has been cloned and genetically placed in a pathway controlling meristem determinacy, very little is known about its molecular mechanisms. Here, the quantitative DGE data were used to investigate putative targets of the RA3 gene. We leveraged functional information available for Arabidopsis and rice (Oryza sativa) and resolved the differential expression of transcription factors (TFs) across a wide range of transcript abundance. The significance of this study is 2-fold. First, our results provide a basis for the analysis of short-read, 3′-targeted expression data using the maize B73 genome as a reference. We demonstrate that quantitative differences in transcript abundance can also be detected by DGE with no prior knowledge of the gene space; therefore, it is applicable to species without sequenced genomes. Second, genetic control of branching, especially in the ear where kernels are borne, has clear relevance to crop improvement programs with respect to seed number and harvesting ability.

RESULTS

Library Construction and Sequencing

To generate digital expression signatures for young maize inflorescences, we used the Illumina Genome Analyzer (GA; first phase) technology for massively parallel sequencing by synthesis. In addition, we used a mutant in the RA3 gene as a developmental perturbation. Immature ears were sampled and hand dissected from field-grown wild-type B73 inbred (Fig. 1A) and ra3 mutant plants introgressed into a B73 background (Fig. 1B). Ears were size selected for uniformity at a growth stage of 2 mm, where expression of RA3 is highest (Satoh-Nagasawa et al., 2006) in the wild type (Fig. 1, C and E) and the very first signs of the mutant phenotype were visible as outgrowths of the spikelet pair meristems (Fig. 1, D and F). We represented the wild-type and ra3 genotypes each with three pools of four to five ears from individual plants. Total RNA was used to construct DGE libraries from each of the six ear samples: three wild-type biological replicates and three ra3 biological replicates. A single technical replicate of a ra3 sample was also run.

Briefly, the DGE technology uses a 3′-targeted sequencing strategy to generate a single 20- or 21-base signature tag from the 3′-end of a given transcript. The length of the tag depends on the restriction enzyme used in library construction. We constructed enzyme-specific libraries for each sample using restriction digests with DpnII and NlaIII. We hypothesized that each enzyme would cleave a given transcript at its 3′-most restriction site and that a dual-enzyme approach would enhance coverage in cases where a restriction site was either absent or within 20 bases of the poly(A) tail. To enable multiplexing of DpnII and NlaIII libraries, we used a custom sequencing primer that incorporated the restriction site at the 5′-end of each read. The specificity of the restriction site thus allowed for library recognition and sorting of reads sequenced concurrently in a single lane. We sequenced each sample in one lane of an eight-lane Illumina GA flow cell.

In total, approximately 28 million filtered, high-quality reads were sequenced from the seven lanes. Custom Perl scripts were used for adaptor trimming and read parsing. Total reads sequenced per individual sample were 3.9 ± 1.1 × 10⁶ (Supplemental Fig. S1A), and about 11% more reads were sequenced from NlaIII libraries than DpnII. We consolidated reads from all seven lanes into 290,000 and 490,000 unique tags from the DpnII and NlaIII libraries, respectively. Approximately half of these nonredundant tags were singlets; however, they only represented 1.5% of total reads sequenced. Singlets were removed from further
analyses on the basis that they likely represent sequencing errors and there is no statistical support for their presence. Read frequencies of unique signature tags represented by two or more reads (consensus tags) spanned over 4 orders of magnitude (Supplemental Fig. S1B).

Mapping Short Reads to the Maize Reference Genome

We used Vmatch (www.vmatch.de) to map unique consensus sequence tags (total of two or more reads from all libraries) to the maize reference genome (B73 RefGen_v1 [Schnable et al., 2009]). The Vmatch algorithm uses enhanced suffix arrays (Abouelhoda et al., 2002) in which a persistent reference index is created allowing for efficient processing time and reduced space requirements. This method performs effectively with DGE data sets, which are reduced in size and complexity since reads are collapsed to unique tags prior to mapping. Other algorithms for large-scale mapping of short reads can also be used with the condensed DGE data, and we have achieved analogous results using Bowtie (http://bowtie.bio.sourceforge.net).

The short-read mapping pipeline used here included two rounds of mapping to the maize reference genome and associated transcript models (Fig. 2). In phase I, we used a stringent requirement for a complete match of the sequence tag. Here, we allowed a given tag to map perfectly up to three places in the genome. Since we expect that 3′ regions of a given gene tend to be unique, tags mapping to four or more locations were considered repeats and removed from further analyses. In this first round of mapping for DpnII/NlaIII tags, 45%/54% mapped to a single location, 6%/8% mapped to two or three individual places in the genome, 5%/5% were considered repeats, and 44%/33% did not map (Table I). To determine whether a portion of these unmapped tags covered splice junctions, we used the transcript models associated with the maize reference sequence (www.maizesequence.org) as a persistent Vmatch index. We recovered an additional 1.7%/4.8% (DpnII/NlaIII) of total tags that mapped to a single location in the transcriptome and 0.4%/0.9% that matched two or three transcripts.

In phase II, a second round of mapping used the remaining 42%/31% (DpnII/NlaIII) of tags that did not map completely to the genome or associated transcript models. Here, we allowed for one mismatch to maximize the recovery of signatures that did not map due to sequencing errors in the reference or polymorphisms retained after introgression of the ra3 mutant. While the one-mismatch tags tended to be distributed uniformly across the genome, we did observe an enrichment of tags flanking the RA3 locus on chromosome 7 that were sequenced exclusively from the ra3 samples and represented by at least 10 reads (Supplemental Fig. S2). Although these made up only 0.2% of all unique mapped tags, they could potentially be used to resolve areas of variation associated with the introgression. After two rounds of mapping, only 14%/13% (DpnII/NlaIII) of all unique tags did not map to the reference maize genome sequence or associated transcripts (Table I). These nonmapped tags most likely represent regions where the reference sequence is incomplete or varies between B73 and the original ra3 mutant line. Only 0.02% of nonmapped tags matched maize chloroplast or mitochondrial genome sequences.

Extracting Gene Information for Mapped Tags

In the next stage of our analysis pipeline, we used the mapping coordinates for tags that matched one to three unique places in the genome and extracted the corresponding gene information. Custom scripts used the Ensembl Perl Application Programming Interface (http://uswest.ensembl.org/info/docs/api) to associate mapped tags with a “working” gene set of 108,745 gene models including evidence-based (86%) and ab initio (14%) predictions (gene build 4a.53; maizesequence.org). The working gene set is a broader, less conservative set of gene models, which include a smaller set of high-confidence, “filtered” gene models (gene build 4a.53; maizesequence.org). We anticipated that by using these working genes, our analysis would not be restricted to well-characterized genes and thus enhance the potential for gene discovery. In order to maximize the inclusion of unannotated untranslated regions (UTRs), the predicted gene space was extended 300 bases on either end. In total, we identified 37,117 working genes (including 22,500 filtered genes) that were associated with at least two read counts in DpnII and/or NlaIII libraries. Of these, 9% were DpnII specific while 21% were found only in the NlaIII data set. We then

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**Figure 2.** Bioinformatics pipeline used to map unique DGE tags to the maize reference genome. In phase I, only perfect matches were allowed. Tags that mapped uniquely up to three places in the genome or associated cDNA models were used in downstream analyses to extract Ensembl-based gene information. Tags that did not map during phase I were subjected to an additional round of mapping (phase II), which allowed for one mismatch along the length of the sequence tag. All tags that mapped to repeat regions of the genome (more than three unique places) were removed from further analyses.
compared total read frequencies to the percentage of mapped tags associated with working gene models, nongenic space, and repetitive regions and to nonmapped tags (Fig. 3). The proportion of tags that mapped to a working gene model increased from approximately 40% at low read counts (two to 10 reads) to 80% at 1,000 reads or more. Overall, there were more of the low-frequency tags sequenced from the Nla III libraries (Supplemental Fig. S1B), and a higher percentage of these mapped to genic regions compared with those from Dpn II libraries. Tags that mapped to repetitive regions represented about 6% of the total in each frequency class (Fig. 3), and the majority of nonmapped tags were low copy (50% and 40% for Dpn II and Nla III libraries, respectively). We also observed a small proportion of tags in each read count class that mapped to regions of the genome where no gene model was called. When mapping from phase II (one mismatch allowed) was considered, an additional 2,936 working genes were identified; however, most of these were recovered from low-frequency tags (Supplemental Fig. S3).

Since the number of genes recovered (37,117) was only 15% of the total number of mapped tags, multiple signature tags were likely associated with a single gene. Therefore, we combined consensus sequence tags that mapped to a given gene model to obtain a cumulative read count (Fig. 4A). Only 36% and 24% of the genes identified in DpnII and NlaIII libraries, respectively, were associated with a single signature tag. We expect that multiple tags are due to incomplete restriction enzyme digests during library preparation; however, a portion may represent alternate splice isoforms or polyadenylation variants. The number of tags per gene was distributed over a wider range in the NlaIII data set, with some genes associated with more than 20 tags. This distribution was also observed with an independent maize NlaIII data set constructed and sequenced in a different laboratory (P. Bommert, unpublished data), suggesting that this was not due to technical errors during library construction.

Although multiple tags mapped to a given gene, we expected that the most abundant tags would be those associated with the 3′-most restriction site for each gene. To test this, we used a set of 36,394 full-length maize cDNAs (fl-cDNAs; Alexandrov et al., 2009) as a “golden” reference set of transcript models with

### Table I. Summary statistics from mapping unique sequence tags (represented by two or more reads) to the maize B73 reference genome

| Mapping Class | Phase I | Phase II |
|---------------|---------|----------|
|               | Genome | Transcripts Only | Genome | Transcripts Only |
| **DpnII libraries** |         |           |         |           |
| Total unique tags | 134,656 | 58,746 | 57,503 | 19,431 |
| Unique match | 60,418 (44.9) | 995 (1.7) | 20,525 (35.7) | 111 (0.6) |
| Two to three matches | 8,668 (6.4) | 222 (0.4) | 11,084 (19.3) | 59 (0.3) |
| Repeats | 6,824 (5.1) | 243 (0.04) | 6,463 (11.2) | 4 (0.2) |
| Nonmapped | 58,746 (43.6) | 57,503 (97.9) | 19,431 (33.8) | 19,257 (99.1) |
| **NlaIII libraries** |         |           |         |           |
| Total unique tags | 237,005 | 78,662 | 74,034 | 30,597 |
| Unique match | 128,332 (54.2) | 3,794 (4.8) | 26,708 (36.1) | 289 (0.9) |
| Two to three matches | 18,775 (7.9) | 702 (0.9) | 11,583 (15.7) | 137 (0.4) |
| Repeats | 11,236 (4.7) | 132 (0.2) | 5,146 (7.0) | 21 (0.1) |
| Nonmapped | 78,662 (33.2) | 74,034 (94.1) | 30,597 (41.3) | 30,150 (98.5) |

a(% of Total) represents the portion of tags mapped from total tags subjected to each stage of mapping.

**Figure 3.** Mapping results for unique consensus tags (represented by two or more reads) and distribution by total read count. Coordinates of mapped tags from phase I were used to associate sequence reads with a working set of maize gene models (gene build 4a.53; maizequence.org). Tags were classified as mapping to working gene models or nongenic regions, repeats, or not mapping at all. Results are displayed as consensus tags, grouped by total read count across all samples. In both DpnII (top) and NlaIII (bottom) data sets, nonmapped tags tended to be low copy (two to 10 reads), while the largest portion of tags at read frequencies greater than 100 tended to be associated with gene models.
mostly complete 3'-UTRs. We simulated DpnII and NlaIII digests in silico and matched the predicted DGE tags from the resulting fragments to our consensus set of unique sequence tags. In total, 67% and 74% of the total expressed gene set used in DpnII and NlaIII data sets were grouped according to number of unique tags that map to them. Digests were simulated in silico with DpnII and NlaIII on a golden set of fl-cDNA models. Positions of predicted 20- to 21-nucleotide fragments that matched unique DGE tags were used to estimate frequency (shown as percentage) of the most abundant tag per fl-cDNA generated by each possible site from the 3'-end.

Determining Differential Gene Expression

To compare gene expression profiles for the wild type and mutant, we first used cumulative counts of all consensus tags mapping in sense to a given working gene model (Supplemental Fig. S5). Initially, only tags that mapped completely to the genome and/or transcript models (phase I) were used, and data from DpnII- and NlaIII-generated data sets were analyzed separately. Since we included tags that mapped to two or three locations in the genome, we applied a scoring system to normalize read distribution across lanes to avoid the skewing of very-high-frequency sequences by plotting raw read counts for the most abundant tags encountered in DpnII and NlaIII libraries (Supplemental Fig. S6).

We used edgeR (empirical analysis of digital gene expression in R; Robinson and Smyth, 2008; Robinson et al., 2010), a software package available from Bioconductor (Gentleman et al., 2004), to normalize for tag distribution per library and determine significance values for differentially expressed genes. The edgeR algorithm uses an empirical Bayes analysis to improve power in small sample sizes (Robinson and Smyth 2007, 2008; Robinson et al., 2010). This accounts for biological and technical variation and has been implemented for tag-based data sets where small numbers of replicates are tested and SE values disperse farther from the mean at low versus high levels of expression (Robinson and Smyth 2008; Babbitt et al., 2010). Based on a cutoff of at least nine reads per gene, our statistical analyses included 20,250 and 22,130 genes for DpnII and NlaIII data sets, respectively (Supplemental Data Set S1). From these, we identified 660 and 303 differentially expressed genes, respectively, with false discovery rate-corrected P values less than 0.05 (Fig. 5A; Supplemental Fig. S7). A smaller number of significantly different genes identified in the NlaIII data set is likely due to more variation observed among NlaIII samples and small sample sizes. Among all differentially expressed genes (P < 0.05), 629 were up-regulated in the ra3 mutant while 249 were down-regulated. Many of the genes showing the most significant differences in expression have not been characterized in maize or in related species (Supplemental Table S2).

Although 74% of the total expressed gene set used in the edgeR analysis was present in both DpnII and
NlaIII data sets, only 9% of these genes had significant \( P \) values \(<0.05\) in each of the two data sets (Fig. 5A; Supplemental Table S3). Although the remaining 65% (675 genes) had significant \( P \) values exclusively in DpnII or NlaIII data sets, the majority of these (97.4%) showed the same trend (i.e. either up- or down-regulated in the ra3 mutant) in both data sets (Fig. 5B). As expected, a larger proportion of genes that were highly significant \( P < 1.0e^{-08} \) in one of the two data sets also showed significant expression differences in the other (Fig. 5A). We used quantitative real-time PCR (Q-PCR) with independently collected RNA samples to test whether expression differences were reproducible for a subset of genes. Significant differences were validated for 80% of the genes tested using Q-PCR (Fig. 5C).

**Expression Profiling with No Prior Knowledge**

To determine differences in transcript abundance independently of prior information on gene models, we carried out statistical testing using edgeR for each mapped tag separately (Supplemental Data Set S2). In this analysis, we used tags that mapped completely to
the genome and were independently represented by at least nine reads, rather than cumulative counts per gene. We identified 364 unique tags from DpnII libraries and 294 tags from NlaIII libraries that showed significant differences in frequency between wild-type and ra3 mutant samples. These tags were grouped into four hierarchical classes based on level of significance (where class I included tags with most significant \( P \) values and class IV included tags with least significant \( P \) values; all classes were <0.05). Their individual read frequencies were then plotted according to tag rank order (Fig. 6). Mapping the tags back to their respective gene models resulted in comparable sets of differentially expressed genes from DpnII and NlaIII data sets, as observed with cumulative counts (data not shown). Results from this analysis showed that although more counts per tag provided greater power for statistical testing, we could also capture significant differences for low-frequency tags with read counts just above 10.

These data indicate that differential gene expression can be analyzed without prior knowledge of gene models and that significant differences can be detected for low-abundance transcripts. Therefore, with ongoing upgrades in throughput for Illumina and other sequencing technologies, the ability to multiplex DGE samples can dramatically improve cost and time efficiency. In addition, these analyses provide a more unbiased approach and an advantage over microarray design, since existing gene models may not be complete or capture all transcript variants. We further tested whether tags that mapped to unannotated regions of the genome were of potential biological significance. Of those individual mapped tags with significant differences in frequency between the wild type and mutant (Fig. 6), 37 DpnII and 25 NlaIII unique tags did not associate with working gene models. We used mapping coordinates of these 62 unique tags to cluster those that mapped within 5 kb of each other. This identified four unique regions where these differentially expressed tags clustered (Supplemental Fig. S8). Among these, a 554-nucleotide region on chromosome 8 and a 488-nucleotide region on chromosome 4 were associated with tags expressed only in ra3 samples. Although no gene model has been called in either of these regions, available RNA-seq data for maize (maizesequence.org; Schnable et al., 2009) provide additional evidence for expression. A 57-nucleotide region on chromosome 5 and a 531-nucleotide region on chromosome 7 were each associated with two differentially expressed tags and appear to be unannotated 3’-UTRs to adjoining genes (Supplemental Fig. S8). This clustering method could also be done with all mapped tags to obtain cumulative read counts in the absence of predicted gene models.

Resolving TFs across a Wide Range of Abundances

A primary objective was to identify genes that encode TFs and to determine potential ranges for their detection. To test this, we used information from the Ensembl Compara gene trees (Vilella et al., 2009) at maizesequence.org and gramene.org (Liang et al., 2008) to retrieve putative orthologs of maize genes in our expressed set. We then queried known Arabidopsis TFs in the Database of Arabidopsis Transcription Factors (http://datf.cbi.pku.edu.cn/) and between both DpnII and NlaIII data sets identified 479 maize genes with sequence similarities to Arabidopsis TFs. Quantitative analysis of their expression profiles indicated that TFs are expressed over a wide range of transcript abundances, spanning over 4 orders of magnitude in young inflorescence tissue (Fig. 7; Supplemental Data Set S3). Of the 479 putative TFs, 27 were differentially expressed \( (P < 0.05) \) based on
edgeR statistical analysis. We further interrogated the remaining differentially expressed gene set for additional TFs using gene ontologies, InterPro domains, and known maize annotations.

Together, these analyses identified a total of 75 putative TFs, which were differentially expressed over a wide range of abundances (Fig. 7; Supplemental Table S4). A subset presented in Table II includes members of TF families based on InterPro signatures (www.ebi.ac.uk/interpro) associated with functions in development and meristem maintenance or identity (NAC, YABBY, GRAS), while others have roles in hormone-mediated signaling by auxin (AUX/IAA), brassinosteroids (BES, BIM), or ethylene (AP2/ERF). Among the 75 differentially expressed TFs, nine were characterized as AP2/ERF family proteins (PF00847) based on InterPro and PFam classifications (Fig. 7).

DISCUSSION

In this study, we developed and tested a framework for analysis of short-read, sequence-based expression profiles using Illumina’s DGE technology and the first assembled maize reference genome (B73 RefGen_v1 [Schnable et al., 2009]). Our results demonstrate that deep sequencing of 20- to 21-nucleotide DGE tags can be used to successfully resolve genome-wide expression profiles in maize and detect differences in transcript abundance over a broad dynamic range. In our test case, which compared three pooled samples each of wild-type and *ra3* ears, we identified 37,117 expressed maize working genes (including 22,700 high-confidence filtered genes) from six DGE libraries, each sequenced in a single lane of an Illumina GA flow cell. Of these, 67% were represented by sense transcripts with nine or more reads and were used to test for significant differences in transcript abundance between the wild type and *ra3* mutants. Results from these expression analyses provide testable hypotheses for resolving regulatory and biochemical processes contributing to maize inflorescence architecture via the RA pathway.

Evaluation of DGE-Based Analysis with the Maize Reference Genome

One objective of this work was to evaluate the performance of DGE as a high-throughput method for genome-wide transcript profiling in maize. As deep sequencing technologies continue to develop, their ability and/or efficiency for addressing specific research questions will be based on thresholds that exist for read length, depth of coverage, sequence specificity, and cost. One limitation of the Illumina tag-based DGE platform is the short read length (20–21 nucleotides); however, its exceptional throughput at a lower cost promotes DGE as a candidate for use in large-scale expression profiling experiments. We speculated that the availability of a sequenced maize genome would improve our ability to map the short DGE tags. From the conservative mapping phase (phase I) of our DGE analysis pipeline, we were able to map 51% of unique sequence tags to a single location in the maize genome. Although 35% of unique tags did not map in phase I, our results are consistent with those of ’t Hoen et al. (2008), who showed that 41% of DGE tags could not be mapped in mouse. By performing an additional round of mapping (phase II, one mismatch allowed), we were able to recover 63% of these nonmapped tags. The remaining 14% were most likely due to incomplete regions of the reference sequence and variation due to introgression.

Other studies using DGE (’t Hoen et al., 2008; Morrissy et al., 2009) and MPSS (Meyers et al., 2004; Nobuta et al., 2007) included only those tags that mapped unambiguously to the reference sequence in their analyses. One feature of the Vmatch mapping software used here is the ability to view all redundant matches to the genome. In this work, tags that mapped perfectly to two or three individual loci represented 9% of the total. These were included in our analyses with the expectation of recovering information on paralogous genes. For example, for a set of AP2/ERF family members, we could successfully resolve closely related paralogs in the maize genome and quantify their unique transcript profiles. Our confidence for detecting paralogous loci was improved by combining...
tags from DpnII and NlaIII data sets that associated with a given gene model (Supplemental Table S5).

Similar to observations in this work, previous studies with tag-based profiling methods using either the DpnII (Meyers et al., 2004) or the NlaIII (‘t Hoen et al., 2008; Babbitt et al., 2010) enzymes also showed that multiple tags tended to associate with a single gene model. Although these have been suggested to represent polyadenylation variants, we expect, based on the high frequencies and wide distribution of tags, that many are due to incomplete restriction enzyme digestion during library preparation and/or enzyme biases for specific cut sites. In support of this, analysis of the biological replicates in our study showed that read frequencies of individual tags varied among replicates to a larger degree than the cumulative counts for a given gene (Supplemental Fig. S5). Profiling with cumulative counts also allowed for the inclusion of more genes in our statistical analysis, since a cutoff of nine reads was imposed as a threshold for detection. In addition, our analysis using the full-length cDNA models indicated that the 3'-most tag was not always the most abundant signature for a gene. This was more commonly observed with the NlaIII enzyme, which further suggests that multiple signatures per gene were not due to variation in transcript structure.

Based on these observations, we used a cumulative count of tags mapping to a given gene, as opposed to previous DGE and MPSS studies that used a predicted 3'-tag database for detecting individual transcripts (Meyers et al., 2004; ‘t Hoen et al., 2008; Morrissy et al., 2009). Relying on the latter method in a complex genome such as maize, which is in the early stages of annotation, could result in a loss of informative expression data. Accordingly, recent work using RNA-seq data to improve gene models in human identified

### Table II. Differentially expressed maize genes were identified as putative TFs

| Maize Gene ID | Annotationa | TF Family | Read Frequencyb | LogFCc | P Value d |
|-------|-------------|-----------|-----------------|--------|--------|
|       |             |           | Wild Type       | ra3    |         |
| 1     |             |           |                 |        |        |
| 2     |             |           |                 |        |        |
| 3     |             |           |                 |        |        |
| 4     |             |           |                 |        |        |

*aAnnotations are based on Ensembl gene descriptions at maizesequence.org, gene build 4a.53.  
*bRead frequency is average read count ± SE (reads per million) for the three biological replicates in wild-type and ra3 samples.  
*cLog fold changes from edgeR analysis of differential gene expression.  
*dCorrected P values (false discovery rate of 5%) from edgeR analysis of differential gene expression. If P < 0.05 in both enzyme libraries, values for DpnII and NlaIII are shown, respectively.

As mentioned before, the statistics calculated from the RNA-seq data to improve gene models in human identified
extensive unannotated UTRs (Pickrell et al., 2010). In our analysis, although we expect to lose the resolution of alternate transcripts, such as 3′-RNA processing variants, combining tags on a per-gene basis, including tags mapping within 300 bases of a predicted UTR, provides more comprehensive expression analyses for poorly annotated genes. Combining DGE data sets with long-read or paired-end RNA-seq approaches would likely improve confidence for identifying alternatively spliced transcripts.

Analysis of Enzyme-Specific Data Sets

One ambiguity of sequencing methods that use restriction enzymes in library construction is the potential bias and/or efficiency of enzymes for specific sequences (Siddiqui et al., 2006). Our study represented the first direct comparison, to our knowledge, of two different enzymes across a set of biological replicates. On average, there were 1.7 times more unique tags sequenced from NlaIII libraries compared with DpnII. This may be expected, since NlaIII cuts approximately 1.5 million more times in the maize genome, and approximately 132,000 more times in the transcriptome, than DpnII. In addition, NlaIII generated more unique tags per gene model overall (in some cases up to 20 individual tags per transcript) and more primary signature tags from 3′-most restriction sites when compared with DpnII. These data suggest that processing of alternate cut sites is not random between the two enzymes and that DGE libraries generated from a single enzyme may be prone to biases.

Based on observations from unrelated experiments that NlaIII produces more tags per gene (P. Bommert and M. Regulski, unpublished data), it is possible that noise from partial restriction enzyme digestions can skew expression profiles and dilute biologically relevant information. Other factors, such as a slight G+C bias in the NlaIII library (Supplemental Fig. S9), amplification biases prior to sequencing, or the general instability of the NlaIII enzyme, may contribute to the limited overlap of differentially expressed genes between DpnII and NlaIII data sets. This could further be explained by the small sample sizes typically used in sequence-based profiling experiments, which are subject to false positives, and more variation among NlaIII samples, presumably due to technical bias during library construction. However, in this study, our approach to analyze DpnII and NlaIII data sets independently enhanced the power to detect highly significant changes in gene expression while decreasing false positives.

Other sequence-based expression studies have found that analyses of low-copy transcripts were often unreliable, even in the absence of enzyme-based library construction (Marioni et al., 2008; Mortazavi et al., 2008; Fahlgren et al., 2009). Here, although we observed less variation between replicates for highly expressed genes, differences in expression trends between the two data sets did not seem to correlate with total read count (data not shown). Overall, despite variations between the enzyme-specific data sets, we showed that the dual-enzyme approach provided expression data on a more complete panel of genes as well as validation for a high-confidence set of genes identified as significant from both data sets. The latter was especially true for genes with highly significant expression differences between test groups. Furthermore, we showed that expression profiles identified in both DGE data sets could be experimentally validated by Q-PCR.

Applications for Functional Analyses with DGE Data

After identifying differentially expressed genes, the next step is to ask whether these genes reveal functionally relevant information. However, most genes with significant differences in transcript abundance were largely uncharacterized in maize or closely related plant species. This is due, in part, to the fact that functional ontologies used to classify genes (i.e. Gene Ontology [GO] and Pfam) are primarily based on bacterial and animal models, and many plant-specific genes have not been functionally annotated. Of the total expressed genes that were used in statistical testing (22,267 and 24,997 from DpnII and NlaIII data sets, respectively), only 48% were associated with GO terms. Consequently, this impacted our ability to resolve significant enrichment for gene ontologies in the DGE data sets. However, we found overrepresentation of intercellular (Cellular Compartment, GO:5622, \( P = 1.94 \times 10^{-16} \)) and RNA binding (Molecular Process, GO:3723; \( P = 1.84 \times 10^{-02} \)) in the differentially expressed gene set. These results are consistent with the predicted roles for RA3 in cell-to-cell signaling and gene regulation (Satoh-Nagasawa et al., 2006). We anticipate that as expression data sets for maize are generated, functional annotations will improve through the integration of metadata and the curation of coexpressed genes and pathways (Horan et al., 2008).

Although a large proportion of genes that showed significant transcriptional changes have not been characterized in maize, we were able to leverage known functional information from Arabidopsis and rice to identify putative classes of metabolic and regulatory genes. For example, rice genes have been associated with biochemical pathways, and we used the ricecyc pathway tool (www.gramene.org; Liang et al., 2008) to determine whether differentially expressed genes were assigned to common metabolic pathways. For this, we used a significance threshold of \( P < 0.08 \) for differential expression in order to increase the coverage of pathways and identified 781 putative rice orthologs using the Ensembl Compara gene trees (maizesequence.org, gramene.org; Vilella et al., 2009). Of these, 67 (54 up-regulated and 13 down-regulated in ra3 mutants) could be mapped onto 97 specific metabolic pathways (Supplemental Table S6).

The RA3 gene is expressed in a narrow band subtending the maize spikelet pair meristems during early
inflorescence development (Satoh-Nagasawa et al., 2006). As a TPP, it is possible that a mobile signal, such as a sugar, could be mediating RA3’s control of axillary meristem cell fate (Rolland et al., 2006). We observed that many of the differentially expressed genes that could be mapped onto metabolic pathways were associated with primary carbohydrate biosynthesis and degradation, respiration, and energy production as well as redox and nitrogen cycling processes (Supplemental Table S6). As expected, trehalose biosynthesis was represented in the differentially expressed set. Expression of the RA3 gene was significantly down-regulated in the mutant, as was a trehalose phosphate synthase (GRMZM2G077659). In contrast, an uncharacterized gene in maize (GRMZM2G151044) with sequence similarity to a TPP in Arabidopsis was up-regulated in the mutant, possibly as compensation for reduced RA3 levels (Fig. 5C; Supplemental Fig. S10).

Disruption of trehalose biosynthesis in the ra3 mutant could have global affects on the sugar status of the cell due to altered Glu-6-P and trehalose-6-phosphate levels. We found that pathways for transient starch degradation (Smith et al., 2005) and downstream reactions that utilize hexoses as substrates (i.e. Glu-6-P) were also represented in the differentially expressed set. These included glycolysis and the oxidative pentose phosphate pathway, both of which generate reducing power in the form of NAD(P)H. The intermediates generated by the reaction of these enzymes represent potential signals that report the sugar, redox, or adenylate status of the cell (Supplemental Fig. S10). In addition, a number of genes that encode enzymes with oxidoreductase activities were differentially expressed and were primarily up-regulated in the mutant. These activities also have the potential to generate signals. For example, previous work has implicated the trehalose intermediate trehalose-6-phosphate as mediating the redox regulation of a key starch biosynthetic enzyme (Kolbe et al., 2005).

Advantages of DGE for Genome-Wide Transcript Profiling

We found that DGE can be used to effectively determine genome-wide transcriptional changes. Aside from the prospect of using DGE in species where commercial arrays are not available, this method enables transcript profiling independent of prior knowledge of gene models, a considerable advantage over microarrays. This is especially relevant in cases where gene annotations are not complete. By mapping DGE tags and comparing abundances independently of gene models, we could resolve novel regions of expression in the maize genome. Some of these were specific to the ra3 mutant, suggesting that analyses limited to existing gene models could exclude tissue- or mutant-specific transcripts. We also showed that even with short 20- to 21-nucleotide tags, we could delineate differential expression of closely related paralogs, which is limited in arrays due to cross-hybridization.

The DGE method also provides strand specificity, which is an advantage even over current RNA-seq protocols. Previous work showed that differential expression of antisense transcripts and S-AS pairs was common in maize (Ma et al., 2006). In human cell lines, evidence for shifts in the ratios of sense to antisense transcripts and S-AS pairs has indicated possible antisense-based regulation of developmental and disease processes (Chen et al., 2005; Morrissy et al., 2009). Consistent with results from other recent DGE studies, which resolved genome-wide expression of previously uncharacterized antisense transcripts (Morrissy et al., 2009; Babbitt et al., 2010), we identified a number of S-AS pairs with ratio changes between wild-type and ra3 mutant samples. Further analyses will be needed to test the biological significance of the antisense transcripts; however, their detec-
tion provides a more accurate quantification of gene expression.

The ability to analyze strand-specific DGE data independent of gene models also enabled the detection and quantification of primary microRNA transcripts (pri-miRNAs). Based on computationally predicted miRNA hairpin structures (pre-miRNAs) and a set of PCR-RACE-validated pri-miRNA sequences identified by Zhang et al. (2009), we identified 14 pri-miRNAs (in 10 different families) represented by 10 or more reads in our DGE libraries (Supplemental Table S7). One abundant pri-miRNA, for miR159a, was significantly reduced in the ra3 mutant; however, we observed no differential expression of its predicted target genes (Zhang et al., 2009), presumably due to miRNA redundancy. Resolution of miRNA abundances and correlations with expression differences in putative targets would be enhanced through the integration of parallel small RNA libraries with these data.

In addition, the tag-based nature of DGE technically generates only one read per transcript and thus improves cost efficiency by reducing redundancy. In this study, we multiplexed two enzyme-specific libraries in each lane of a flow cell using Illumina’s first-phase GA. Although we achieved sufficient resolution of a large panel of TFs and differential expression profiles for low-frequency transcripts, the rapidly advancing technology currently generates estimated read depths of 3-fold higher than shown here. Given this current throughput, a multiplexed approach to concurrently sequence biological replicates and/or treatments would further reduce cost, time, and potential technical biases.

CONCLUSION

Digital gene expression profiling by high-throughput sequencing of 20- to 21-nucleotide tags revealed quantitative changes in transcript abundance on a genome-wide scale. Results from our DGE analyses in maize showed that we could effectively identify differentially expressed genes across a wide range of transcript abundances. Cumulative counts of tags that mapped to predicted gene models enabled the identification of functionally interesting genes and gene families with altered expression in ra3 mutants. Our parallel analysis independent of gene models demonstrated that expression profiling was not limited by prior knowledge, thus promoting DGE as a platform for exploratory studies in species with nonsequenced genomes and for gene discovery. We also used the DGE data to resolve sense and antisense transcripts, distinguish between closely related paralogs, and identify unannotated genes and UTRs. Our approach used two enzymes to generate independent data sets that were multiplexed in a single lane of an Illumina flow cell. This provided a cost-effective method for orthogonal validation of genome-wide expression signatures and improved our coverage of the gene space. Our analyses, applications, and findings used here to interrogate the maize transcriptome and identify expression signatures underlying an agriculturally important trait are readily translated to other systems.

MATERIALS AND METHODS

DGE Library Construction and Sequencing

Field-grown maize (Zea mays) B73 and ra3 plants were collected approximately 7 weeks after planting, and 2-mm ears were hand dissected and immediately frozen in liquid nitrogen. The ra3 allele used here, ra3-f2, results from an insertion, which leads to a frame shift. The ra3 mutant was introgressed into B73 for five generations. RNA isolation, library construction, and sequencing were carried out at Pioneer Hi-Bred in Johnston, Iowa. Here, 500 to 2,000 ng of DNaseI-treated total RNA was used in library construction: double-stranded cDNAs were synthesized using oligo(dT) beads (Invitrogen). The cDNAs were then digested with an anchoring restriction enzyme (NlaIII or DpnII) and ligated to an Illumina-specific adapter, Adapter A, containing a recognition site for the type IIS restriction enzyme Mmel (New England Biolabs). Following Mmel digestion and dephosphorylation with shrimp alkaline phosphatase (USB Corp.), cDNAs were purified and a second Illumina adapter, Adapter B, containing a 2-bp degenerate 3’ overhang, was ligated. Tags flanked by both adapters were enriched by PCR using Phusion DNA polymerase (Finzymes) and Gex PCR primers 1 and 2 (Illumina) following the manufacturer’s instructions. The PCR products were run on a 12% PAGE gel, and the 85-bp DNA band was excised and purified using a Spin-X filter column (Costar) followed by ethanol precipitation. The DNA quality was assessed and quantified using an Agilent DNA 1000 series II assay, and the DNA sample was diluted to 10 ng. Cluster generation and sequencing were performed on the Illumina cluster station and Genome Analyzer (Illumina) following the manufacturer’s instructions. Raw sequences were extracted from the resulting image files using the open source Firecrest and Bustard applications (Illumina). The NlaIII reads were 21 bases long, since the enzyme cut site (CATG) overlapped with the Mmel binding site [TCC(G/A)AC] by 1 base in the sequencing primer. The additional base was later added to the 5’-end of each read in silico.

Mapping Pipeline and Extraction of Gene Information

We used Vmatch (www.vmatch.de) large-scale sequence analysis software to map the collapsed reads to the maize reference genome. The Vmatch feature was used to construct persistent indices for each of the 10 unmasked, assembled maize chromosomes and for an additional chromosome 0, which includes nonassembled sequence (B73 RefGen_v1). For each of the 11 indices, the unique DpnII and NlaIII tags were queried for complete matches to all possible 20- and 21-mer sequences, respectively, using the Vmatch algorithm. This included both sense and antisense matches, and strand information for each tag was retained. In phase II of the mapping pipeline, an editing distance of 1 was used to allow for a single mismatch, insertion, or deletion. Mapping data from all indices were parsed together, and results from DpnII and NlaIII libraries were analyzed independently. Tags that mapped uniquely up to three places in the maize genome were used to extract gene information using the Ensemble Application Perl Interface (http://uswest.ensembl.org/info/docs/api). A working gene set was used (gene build 4a.53; maizesequence.org), and for each gene model, the gene space was computationally extended by 300 nucleotides at both 5’- and 3’-ends to maximize the capture of complete UTRs. When we used a filtered set of 32,540 high-confidence maize gene models (build 4a.53; maizesequence.org), 22,700 genes were identified, of which 79% were found in both data sets and 6% and 14% were DpnII and NlaIII specific, respectively.

Strand Determination and Cumulative Read Counts

For each gene model, read counts associated with tags mapping in sense and antisense orientations were combined separately. Ratio changes for S-AS transcription of a given gene between the wild type and mutant were calculated based on analyses specified by Morrissy et al. (2009).

To determine consistency among libraries, genome-wide expression values (normalized to reads per million) were compared by pair-wise correlations of all libraries. Technical replicates of the same biological sample showed exceptional correlation in both DpnII and NlaIII libraries (Spearman $r^2 = 0.998$; Supplemental Fig. S5A). Combining all reads from multiple tags mapping to a given gene improved correlations among biological replicates.
($r^2 = 0.963-0.861$) compared with plotting individual tag frequencies alone ($r^2 = 0.907-0.552$; Supplemental Fig. S1B). Before determining a cumulative count per gene, we applied a scoring convention where a single read count was kept as 1 if a tag mapped to one place in the genome, 0.5 if it mapped to two locations, and 0.33 if it mapped three times.

**Significance Testing**

To compare gene expression profiles between wild-type and ra3 mutant samples, we used the 26,663 and 30,746 genes identified from sense tags in phase I mapping of DproII and NlalII libraries, respectively. The edgeR package (www.bioconductor.org/packages/2.3/bioc/html/edgeR.html) adjusts for differences in library size; therefore, raw read counts per gene (or per tag) are directly used as input. One of the NlalII mutant samples, ra3-3, was highly variable when compared with all samples (Supplemental Fig. S5, B and C). This was most likely due to technical variation during library construction, since the DproII ra3-3 correlated strongly with other biological replicates. After evaluation of the results from statistical tests, NlalII ra3-3 was removed from the gene expression analyses presented here. We used a moderated, gene-wise dispersion analysis for both DproII and NlalII data sets separately with a weighted prior of 100. We applied a cutoff of nine reads for each gene, which reduced the set to 20,250 and 22,130 genes for DproII and NlalII libraries, respectively. Our significance threshold for differential expression was $P < 0.05$ after correction using a Benjamini-Hochberg false discovery rate of 5%.

**Quantitative Reverse Transcription-PCR Analyses**

Total RNA samples for Q-PCR analyses represented three biological replicates of B73 and ra3 ears and were comparable to those used for the DGE library construction. RNA integrity was assessed on an Agilent BioAnalyzer using a Nano Chip (Agilent 6000 Nano kit 5067-1511) according to the manufacturer’s protocol. A total of four technical replicates were run for each RNA sample per assay using the ABI7900 instrument (ABI; thermal cycling conditions were 50°C for 3 min [reverse transcription step], 95°C for 5 min [initial melt], and then 40 cycles of 94°C for 15 s and 60°C for 1 min). Specificity of each assay was determined by computer database homology searches. The linear dynamic range was determined using a standard curve generated from 1, 0.5, and 0.25 ng of RNA from a single replicate in each assay. Cycling conditions were 50°C for 15 s and 60°C for 1 min). Pair-wise correlations between normalized DGE libraries.

**Supplemental Data**

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

**Supplemental Figure S1.** Distribution of consensus tags and read counts.

**Supplemental Figure S2.** Alignment of one-mismatch tags to the genome.

**Supplemental Figure S3.** Mapping results comparing phase I with phase II mappings.

**Supplemental Figure S4.** Distribution of S-AS ratio changes.

**Supplemental Figure S5.** Pair-wise correlations between normalized DGE libraries.

**Supplemental Figure S6.** Top 10 most abundant tags sequenced in DproII and NlalII libraries.

**Supplemental Figure S7.** Smear plots from the edgeR-based analysis of gene expression.

**Supplemental Figure S8.** Regions of novel gene expression.

**Supplemental Figure S9.** Histogram of G+C content.

**Supplemental Figure S10.** Schematic of differentially expressed genes overlaid on pathways adapted from ricecyc.

**Supplemental Table S1.** Genes with S-AS ratio changes greater than 1.5-fold.

**Supplemental Table S2.** Genes with the most significant changes in transcript abundance.

**Supplemental Table S3.** All differentially expressed genes and their corresponding descriptions.

**Supplemental Table S4.** All putative TFs identified as differentially expressed in DproII and/or NlalII data sets.

**Supplemental Table S5.** Coordinates for individually mapped tags to AP2/ERF paralogs.

**Supplemental Table S6.** Genes associated with biochemical pathways based on ricecyc assignments.

**Supplemental Table S7.** miRNA precursors identified in the DGE data set.

**Supplemental Data Set S1.** All genes used in edgeR expression analysis and resulting fold changes and P values.

**Supplemental Data Set S2.** Individual signature tags used in edgeR expression analysis and resulting P values.

**Supplemental Data Set S3.** Maize identified as TFs based on sequence similarity to Arabidopsis genes.

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