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Abstract
All above-ground plant organs are derived from shoot apical meristems (SAMs). Global analyses of gene expression were conducted on maize (*Zea mays* L.) SAMs to identify genes preferentially expressed in the SAM. The SAMs were collected from 14-day-old B73 seedlings via laser capture microdissection (LCM). The RNA samples extracted from LCM-collected SAMs and from seedlings were hybridized to microarrays spotted with 37 660 maize cDNAs. Approximately 30% (10 816) of these cDNAs were prepared as part of this study from manually dissected B73 maize apices. Over 5000 expressed sequence tags (ESTs) (about 13% of the total) were differentially expressed (P < 0.0001) between SAMs and seedlings. Of these, 2783 and 2248 ESTs were up- and down-regulated in the SAM, respectively. The expression in the SAM of several of the differentially expressed ESTs was validated via quantitative RT-PCR and/or *in situ* hybridization. The up-regulated ESTs included many regulatory genes including transcription factors, chromatin remodeling factors and components of the gene-silencing machinery, as well as about 900 genes with unknown functions. Surprisingly, transcripts that hybridized to 62 retrotransposon-related cDNAs were also substantially up-regulated in the SAM. Complementary DNAs derived from the LCM-collected SAMs were sequenced to identify additional genes that are expressed in the SAM. This generated around 550 000 ESTs (454-SAM ESTs) from two genotypes. Consistent with the microarray results, approximately 14% of the 454-SAM ESTs from B73 were retrotransposon-related. Possible roles of genes that are preferentially expressed in the SAM are discussed.

Keywords
shoot apical meristem, global gene expression, laser capture microdissection, 454 sequencing, development, retrotransposon expression

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Global gene expression analysis of the shoot apical meristem of maize (*Zea mays* L.)

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Summary

All above-ground plant organs are derived from shoot apical meristems (SAMs). Global analyses of gene expression were conducted on maize (*Zea mays* L.) SAMs to identify genes preferentially expressed in the SAM. The SAMs were collected from 14-day-old B73 seedlings via laser capture microdissection (LCM). The RNA samples extracted from LCM-collected SAMs and from seedlings were hybridized to microarrays spotted with 37 660 maize cDNAs. Approximately 30% (10 816) of these cDNAs were prepared as part of this study from manually dissected B73 maize apices. Over 5000 expressed sequence tags (ESTs) (about 13% of the total) were differentially expressed ($P < 0.0001$) between SAMs and seedlings. Of these, 2783 and 2248 ESTs were up- and down-regulated in the SAM, respectively. The expression in the SAM of several of the differentially expressed ESTs was validated via quantitative RT-PCR and/or *in situ* hybridization. The up-regulated ESTs included many regulatory genes including transcription factors, chromatin remodeling factors and components of the gene-silencing machinery, as well as about 900 genes with unknown functions. Surprisingly, transcripts that hybridized to 62 retrotransposon-related cDNAs were also substantially up-regulated in the SAM. Complementary DNAs derived from the LCM-collected SAMs were sequenced to identify additional genes that are expressed in the SAM. This generated around 550 000 ESTs (454-SAM ESTs) from two genotypes. Consistent with the microarray results, approximately 14% of the 454-SAM ESTs from B73 were retrotransposon-related. Possible roles of genes that are preferentially expressed in the SAM are discussed.

Keywords: shoot apical meristem, global gene expression, laser capture microdissection, 454 sequencing, development, retrotransposon expression.

Introduction

Embryonic and post-embryonic development of higher plants initiates from meristems. The shoot apical meristem (SAM) is responsible for the development of all above-ground structures of the plant. The maize (*Zea mays* L.) SAM is formed during embryogenesis (Abbe and Stein, 1954; Randolph, 1936) and is maintained until it

differentiates into a reproductive meristem that will produce the male inflorescence (Carles and Fletcher, 2003). The SAM comprises pluripotent stem cells, which divide to regenerate themselves as well as to provide cells to form other organs such as leaves and stems. Genetic studies have revealed dozens of genes involved in the maintenance of the SAM and in organogenesis, many of which are conserved among plant species (Bäurle and Laux, 2003; Carles and Fletcher, 2003). These genetic approaches, however, have limitations. For example, genetic and functional redundancy may mask the phenotype of any single knockout mutation. It is also challenging to analyze via genetic approaches the SAM-specific functions of essential genes, i.e. those whose mutations are lethal. It is therefore unlikely that genetic approaches can be used to define the entire regulatory network of the SAM.

Microarray technology is a powerful tool for analyzing the expression of thousands of genes in a single experiment. In animal systems, microarray technology has been combined with laser capture microdissection (LCM), which permits the isolation of populations of specific cell types from tissue sections (Emmert-Buck *et al.*, 1996; Luo *et al.*, 1999; Simone *et al.*, 1998). Subsequently, this LCM–microarray approach was used to study global gene expression analyses in specific types of plant cells (Cai and Lashbrook, 2006; Casson *et al.*, 2005; Nakazono *et al.*, 2003; Ohtsu *et al.*, 2007; Tang *et al.*, 2006; Woll *et al.*, 2005). Sequencing a pool of cDNAs is another approach for analyzing global gene expression patterns. Recently, 454 Life Sciences (http://www.454.com/) developed a highly parallel sequencing system that yields 25 million bases from a single genomic DNA sample (Margulies *et al.*, 2005). We have combined 454 sequencing technology with LCM to detect the expression of thousands of genes in specific cell types (Emrich *et al.*, 2007).

Here we report the global analysis of gene expression in the SAMs of 14-day-old B73 maize seedlings using LCM coupled with microarrays and LCM–454 sequencing. In the microarray experiment approximately 13% of analyzed expressed sequence tags (ESTs) were differentially expressed between SAMs and seedlings ($P < 0.0001$). Expressed sequence tags that were up-regulated in the SAM included genes encoding regulatory proteins such as transcription factors, chromatin modification factors, and gene-silencing components. Surprisingly, 62 ESTs that consisted of retrotransposon-related sequences were also substantially up-regulated in the SAMs. Using 454 sequencing about 550 000 ESTs were generated from B73 and Mo17 SAM cDNA pools. Retrotransposon-related sequences were also over-represented among these ESTs (454-SAM ESTs). Analysis of the 454-SAM ESTs also uncovered additional SAM-expressed genes. Possible roles for these genes are discussed.

Results

Construction of a SAM-enriched cDNA library and SAM-enriched cDNA microarrays

Prior to conducting microarray experiments a SAM-enriched cDNA library (Apex library) was constructed from manually dissected B73 maize shoot apices (see Appendix S1 in Supplementary material). The Apex library was sequenced using Sanger technology and 31 036 ESTs generated (Apex ESTs). Subsequently, these Apex ESTs were clustered with other maize ESTs (Appendix S1), which resulted in 10 816 Apex EST singletons and contigs (Table S1). Approximately one-third (3503) of these were classified as being 'Apex-unique' in that they did not cluster with non-Apex ESTs. Each of these 10 816 Apex EST singletons and a representative from each Apex EST contig was spotted on our three microarrays (SAM1.0, GPL2557; SAM2.0, GPL2572; and SAM3.0, GPL3538). More than half of the ESTs spotted on our microarrays were derived from meristem-enriched tissues (see Experimental procedures). In addition, these arrays included ESTs derived from several thousand genes that had not been identified via prior EST projects. These custom microarrays are therefore expected to be more suitable for the analysis of global patterns of gene expression in meristems than are alternative maize profiling platforms that are not specifically enriched for meristem-expressed genes.

Preparation of RNA and microarray experiments

To analyze global patterns of gene expression in the maize vegetative SAM, microarray experiments were performed using RNA samples extracted from SAMs versus the above-ground portions of seedlings. Although each seedling includes a SAM, transcripts from the relatively small (200 μm high) SAMs should comprise only a very small fraction of the RNA from seedlings. We therefore expected this comparison to identify genes that were preferentially expressed in the SAM.

Maize SAMs (defined for the purposes of this paper as the SAM *per se* plus plastochron0 [P0] and P1) were collected from 14-day-old seedlings via LCM (Figure 1). The RNA samples extracted from the maize SAMs and from 14-day-old seedlings were amplified prior to labeling with Cy dyes (Table S2). The quality of all amplified RNA (aRNA) samples was checked via RNA gel electrophoresis of aRNA and via reverse transcriptase (RT)-PCR using intron-spanning primers designed to amplify two portions of the maize β-*6 tubulin* (*tub6*) gene (Appendix S1). All the aRNA samples showed smears ranging from 0.2 to 2 kb in RNA gels and yielded RT-PCR products having the sizes expected in the absence of genomic DNA contamination. An example of these results is presented in Figure S1. Labeled cDNA samples derived from

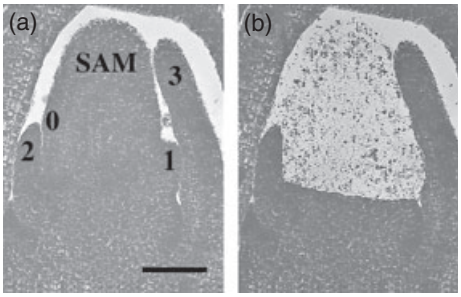

Figure 1. Laser capture microdissection (LCM) of shoot apical meristem (SAMs) from 14-day-old maize seedlings.
A SAM before (a) and after (b) LCM. Leaf primordia are numbered according to their relative developmental ages, wherein P0 (0) corresponds to the incipient leaf forming at the flank of the SAM. Bar: 100 μm.

aRNAs from each of six biological replications were hybridized to our three custom cDNA microarrays (see Experimental procedures). The resulting data were statistically analyzed as described in Experimental procedures.

Differentially expressed genes

The cDNA microarrays include seven genes ('SAM control genes') that have previously been shown to be expressed in the maize SAM and young leaf primordia; Zm *phabulosa*, *rolled leaf1* (Juarez *et al.*, 2004a), *terminal ear1* (Veit *et al.*, 1998), *knotted1*(*kn1*, Vollbrecht *et al.*, 1991), *rough sheath2* (Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999), *zyb14* (Juarez *et al.*, 2004b) and *narrow sheath2* (*ns2*) (Nardmann *et al.*, 2004). The expression patterns of these control genes were analyzed first. With one exception all were consistently up-regulated in the SAM with *P* values of less than 0.01 across the arrays (Table S3). The single exception was *ns2*. This was not unexpected because *ns2* is known to exhibit a restricted expression pattern comprising just eight to ten cells within the lateral domains of the maize SAM (Nardmann *et al.*, 2004).

Next, all the ESTs on the microarrays were analyzed for evidence of differential expression. Those ESTs with *P* values of less than 0.0001 were considered significant, resulting in an estimated false discovery rate of far less than 1% (Appendix S1). Even using this very stringent cut-off, 5031 ESTs (about 13% of the 37 660 informative maize ESTs on the microarrays) were found to accumulate to statistically significant different levels in SAMs versus seedlings. The numbers of genes up- and down-regulated in the SAM relative to seedlings were similar (Table 1). The ESTs on the arrays were derived from various maize organs and tissues. The numbers of up- and down-regulated ESTs varied among these EST sources. Typical examples are presented in Table 1. Four times more up- than down-regulated ESTs were detected among Apex ESTs, whereas the opposite pattern was observed among 2-week-shoot ESTs. Of the 894 up-regulated Apex ESTs, 312 had been classified based on EST clustering as being 'Apex-unique' (Table S1). Fold changes also varied among up- and down-regulated ESTs (Figure 2). Approximately 8% of the up-regulated ESTs exhibited more than a tenfold change (SAM/seedling), whereas as many as 30% of the down-regulated ESTs exhibited more than a tenfold change (seedling/SAM).

Functional annotation of significant ESTs

These differentially regulated ESTs ($P < 0.0001$) were annotated and categorized according to predicted functions (Buckner *et al.*, 2007). Because our primary interest related

Table 1 Expressed sequence tags (ESTs) significantly up- or down-regulated in the shoot apical meristem (SAM)

	Three microarrays combined[a]	Apex ESTs[b]	2-week-shoot ESTs[b]
No. of spots	37 660	10 816	1781
Up-regulated in the SAM	2783 (7.4)	894[c] (8.3)	138 (7.7)
Down-regulated in the SAM	2248 (6.0)	231 (2.1)	551 (31)

Numbers of ESTs significantly ($P < 0.0001$) up- or down-regulated in the SAM relative to the seedling are presented. Parentheses indicate percentages of significant ESTs relative to total number of maize ESTs in each category.
[a]Unique genes among these 37 660 spots were estimated to be 21 721. To estimate this, maize EST contigs (MECs with a 95% cut-off as of March 2006; Fu *et al.*, 2005) that contain EST sequences on the three microarrays were searched. Then maize genomic loci that match these MECs plus EST singletons were searched for within a partial genome assembly of the maize inbred line B73 (MAGI3.1; http://magi.plantgenomics.iastate.edu) that has been estimated to tag between 75% and 90% of the maize gene space (Fu *et al.*, 2005). These genomic loci were considered to be unique genes. If no genomic loci were found, MECs and EST singletons were considered to be unique genes. If only 'unique genes' are considered, 2292 and 1791 genes were up- and down-regulated genes, respectively.
[b]Apex ESTs (Table S1) and 2-week-shoot ESTs (library 947, 2-week-shoots; Table S5) represent only a portion of the ESTs on the three microarrays.
[c]Out of the 894 up-regulated Apex ESTs, 312 were 'Apex-unique' (Table S1).

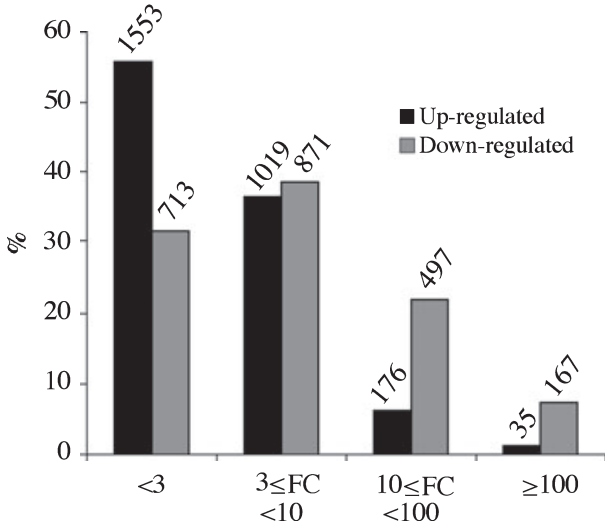

Figure 2. A fold change distribution of the significant expressed sequence tags (ESTs).
Percentages of the up- (black) and down-regulated (gray) ESTs relative to the total number of significant ESTs (2783 up- and 2248 down-regulated; Table 1) in each fold change (FC) category are indicated. Numbers of ESTs in each category are also presented at the top of each bar.

to genes that were up-regulated in the SAM, only those down-regulated ESTs from SAM1.0 and SAM3.0 were annotated (Table 2). Approximately 900 of the up-regulated ESTs were categorized as 'no hits and unknown' (Table 2). Distributions of the up- and down-regulated ESTs differed among the functional categories (Table 2). Categories such as photosynthesis-related were over-represented among the down-regulated ESTs. Categories such as chromatin, cell division and DNA repair were over-represented among the up-regulated ESTs. Out of 217 up-regulated ESTs categorized as chromatin-related, 91 ESTs were annotated as chromatin remodeling. Transcription and gene-silencing categories were also over-represented among the up-regulated ESTs (Table 2). Out of the 234 up-regulated ESTs categorized in transcription, 173 ESTs were annotated as transcription factors. Among the up-regulated ESTs categorized as gene silencing, seven exhibited high similarity to the Arabidopsis *AGO4* gene (encoding an ARGONAUTE protein) (Chan *et al.*, 2004; Zilberman *et al.*, 2003) (Table 3), which is involved in RNA-directed DNA methylation (RdDM) (Bender, 2004; Chan *et al.*, 2005; Wassenegger, 2005). Up-regulated ESTs exhibited similarity to other genes involved in RdDM and heterochromatin formation in Arabidopsis (Table 3). These genes include: *RDR2* (an RNA-directed RNA polymerase), *DCL3* (a Dicer-like RNaseIII-RNA helicase) (Chan *et al.*, 2004; Xie *et al.*, 2004), *NRPD1a* and *NRPD1b* (isoforms of the largest subunit of RNA polymerase IV) (Herr *et al.*, 2005; Kanno *et al.*, 2005; Pontier *et al.*, 2005), and *DDM1* (a SWI2/SNF2-like chromatin remodeling factor) (Jeddeloh *et al.*, 1999; Vongs *et al.*, 1993). Expressed sequence tag CB816774, which is similar to *RDR2,* has recently been shown to be the maize *mop1* gene. *Mop1* encodes an RNA-dependent RNA polymerase, which is required for paramutation and transposon silencing (Alleman *et al.*, 2006; Woodhouse *et al.*, 2006). These genes function to silence repeat sequences such as transposable elements via production of small interfering RNAs (siRNAs) derived from these repeat sequences (Bender, 2004; Chan *et al.*, 2005).

Even given the up-regulation of genes involved in silencing, the transposable elements category was also over-represented among the up-regulated ESTs (Table 2). Sixty-two of the 89 up-regulated ESTs in this category were annotated as retrotransposons. Forty-two of the 62 retrotransposon-related ESTs were up-regulated more than ten-fold and 12 were up-regulated more than 100-fold. Thirty of these up-regulated retrotransposon-related ESTs with highest fold changes are presented in Table 4. The transposable element category also included up-regulated ESTs exhibiting similarity to transposase genes such as *tAT* (Kempken and Windhofer, 2001) and *Mu* (Lisch, 2002) types. The highest fold change among these transposase-like ESTs was 12.

Quantitative RT-PCR and in situ hybridization

The expression pattern of one of the up-regulated retrotransposon-related ESTs (Table 4) and one of the down-regulated ESTs were estimated via semi-quantitative (semi-q) RT-PCR (Appendix S1). Consistent with the microarray results these ESTs exhibited >2000-fold higher and >60-fold lower expression in the SAM than in seedlings, respectively (Table S4). Next, qRT-PCR analyses (Appendix S1) were performed on nine additional ESTs that, based on the microarray experiments, were significantly up-regulated in the SAM ($P < 0.0001$). These nine genes included two additional retrotransposon-related sequences, Cinful and Tekay (Table 4), five ESTs that exhibited similarity to genes involved in RdDM and heterochromatin formation, including maize homologs of *RDR2*, *DDM1* and three maize homologs of *AGO4* (Table 3), and two ESTs annotated as transcription factor genes, including genes encoding a putative MADS-domain transcription factor (MADS; DY401890) and a putative B3 DNA-binding domain transcription factor (B3; DN214788). DN214788 is one of the 'Apex-unique' ESTs (Table S1). At least seven of these genes are more highly expressed in the SAM than in the seedling (Figure 3). For the remaining two genes one of the seedling samples did not yield fluorescence above the detection threshold (see legend to Figure 3).

Transcript accumulation of the maize homolog of *RDR2* (Table 3) was further analyzed via *in situ* hybridization (Figure 4a–f). Transcripts of the *RDR2* homolog are detected uniformly throughout the SAM and youngest leaf

Table 2 Functional annotation of significant expressed sequence tags (ESTs)

Category	No. of up-regulated ESTs (% of total up-regulated)	No. of down-regulated ESTs (% of total down-regulated)
Transcription	**234**[a] **(8.4)**[b]	41 (3.3)
Chromatin	**217**[c] **(7.8)**	3 (0.2)
Metabolism	188 (6.8)	**293 (23)**
Protein fate	188 (6.8)	53 (4.2)
Signal transduction	180 (6.5)	79 (6.3)
Cell division	**166 (6.0)**	11 (0.9)
Translation	138 (5.0)	40 (3.2)
Transport	97 (3.5)	**112 (8.9)**
RNA-binding protein	**97 (3.5)**	11 (0.9)
RNA processing	**94 (3.4)**	12 (1.0)
Transposable elements	**89**[d] **(3.2)**	2 (0.2)
Development	85 (3.1)	34 (2.7)
Cytoskeletal	48 (1.7)	17 (1.3)
Stress-related	47 (1.7)	**123 (9.8)**
Defense	38 (1.4)	**67 (5.3)**
DNA repair	**37 (1.3)**	1 (0.1)
Extracellular matrix/cell wall	29 (1.0)	**38 (3.0)**
Gene silencing	**29 (1.0)**	5 (0.4)
ATPase	21 (0.8)	**35 (2.8)**
Vesicle trafficking	17 (0.6)	**27 (2.1)**
Photosynthesis-related	4 (0.1)	**39 (3.1)**
Respiration	3 (0.1)	**7 (0.6)**
Other[e]	126 (4.5)	51 (4.0)
No hits[f]	251 (9.0)	62 (4.9)
Unknown[g]	663 (24)	250 (20)
Total	3086 (111)	1413 (112)

All significantly up-regulated ESTs (2783) were functionally annotated, whereas significantly down-regulated ESTs were annotated only from SAM1.0 and SAM3.0 (1260). Approximately 11–12% of the ESTs were assigned to more than one category.

[a]173/234 ESTs were annotated as transcription factors.

[b]If there is more than twofold difference between the percentages within a category, the large fold change is given in bold.

[c]91/217 ESTs were annotated as chromatin remodeling.

[d]62/89 ESTs were annotated as retrotransposons.

[e]ESTs that were not assigned to any other category (e.g. 'repeat DNA').

[f]These ESTs had no significant hit (Buckner *et al.*, 2007) when BLASTX searches of GenBank or InterProScan were performed.

[g]These ESTs had hits when BLASTX searches of GenBank were performed, but the hits were annotated as 'unknown protein', 'hypothetical protein', or 'expressed protein'.

primordia (P0 to P2). In slightly older primordia transcripts from the *RDR2* homolog become localized to the abaxial domain of the leaf (i.e. the underside), although transcripts remain evenly distributed at the margins of these primordia. This localized expression pattern is particularly pronounced in P4 and P5 leaf primordia (asterisks in Figure 4b,d). After the P5 stage of leaf primordial development, the transcripts from the *RDR2* homolog accumulate predominately in the margins; no transcripts are detected beyond the P7 stage of leaf development. This expression pattern of the *RDR2* homolog is conserved in other types of shoot meristems, including the ear (axillary) meristem (Figure 4e) and the tassel meristem that forms after the bolting of the SAM into an inflorescence meristem (Figure 4f). *In situ* hybridization analyses were also conducted on an up-regulated EST (DV622566, $P = 5.1 \times 10^{-6}$, fold change 5.6) that exhibited a similarity to a gene encoding a vesicle-associated membrane protein-associated protein (VAP) (Laurent *et al.*, 2000; Skehel *et al.*, 1995), which was categorized in vesicle trafficking (Table 2). Transcripts of the *VAP* homolog form a novel crown-like pattern at the summit of the SAM (Figure 4g,h).

Additional SAM-expressed genes

To further analyze global patterns of gene expression in the maize SAM, we sequenced cDNAs from LCM-collected SAMs. We expected that this approach would uncover additional SAM-expressed genes and thereby supplement our microarray data. Complementary DNA was synthesized using the aRNA samples from LCM-collected B73 SAMs

Table 3 Up-regulated expressed sequence tags (ESTs) exhibiting similarity to genes involved in RNA-directed DNA methylation and heterochromatin formation

Accession no.	*P* value	Fold change	BLASTX results[a] Arabidopsis homolog	*e*-value
CB816774[b,c]	8.0×10^{-5}	120	*RDR2*	8×10^{-69}
DV493642[d]	6.7×10^{-4}	2.8	*DCL3*	5×10^{-10}
DV493575[b,e]	1.1×10^{-5}	16	*AGO4*	1×10^{-57}
DV943274[b,e]	3.2×10^{-5}	7.3		2×10^{-63}
DV492375[b,e]	1.1×10^{-5}	17		1×10^{-78}
DV492666[e,f]	4.2×10^{-6}	11		4×10^{-93}
DN223543[f]	4.2×10^{-6}	7.7		4×10^{-93}
DN209725[e]	2.0×10^{-5}	4.7		5×10^{-168}
DN230051[e]	5.1×10^{-6}	4.6		1×10^{-87}
DN233523	8.5×10^{-5}	4.6	*NRPD1a*	2×10^{-11}
CD651849	6.7×10^{-6}	9.1	*NRPD1b*	2×10^{-19}
DV621159[b,g]	8.6×10^{-8}	9.5	*DDM1*	1×10^{-10}
DV493431[g]	9.1×10^{-5}	18		1×10^{-10}
DV622260[d,g]	1.1×10^{-3}	7.5		1×10^{-10}
DN206716	8.0×10^{-6}	5.5		3×10^{-123}

[a]BLASTX searches were performed using the NCBI nr database on 8 September 2006. Maize EST contigs (MECs with a 98% cut-off as of March 2006; Fu *et al.*, 2005) that contained these EST sequences were used for the BLASTX searches except in the case of DV493642 for which no MEC was available.
[b]Quantitative RT-PCR analysis was performed (Figure 3).
[c]*In situ* hybridization analysis was performed (Figure 4).
[d]Although these genes exhibit highly significant differences in expression their *P* values are >0.0001.
[e]Although highly similar, these ESTs clustered in different MECs.
[f]These ESTs clustered in the same MEC.
[g]These ESTs clustered in the same MEC. DV493431, DV622260, and DV621159 each includes the primer sequences used to amplify the maize *DDM1* homolog (Appendix S1).

used for the microarray experiments. This cDNA was sequenced by 454 Life Sciences (Margulies *et al.*, 2005). In this experiment 260 736 high-quality sequences (454-SAM ESTs) were obtained with an average size of 101 bp after the removal of polyA/T tails. A technical description of these 454-SAM ESTs has been given previously (Emrich *et al.*, 2007). When these ESTs were aligned to a partial genome assembly of the maize inbred line B73 (MAGI3.1; http://magi.plantgenomics.iastate.edu) that has been estimated to tag between 75 and 90% of the maize gene space (Fu *et al.*, 2005), the 454-SAM ESTs aligned to about 25 800 unique MAGI genomic contigs (Emrich *et al.*, 2007).

Retrotransposon-related 454-SAM ESTs

The microarray experiment indicated that 62 retrotransposon-related sequences were strongly up-regulated in the SAM (Tables 2 and 4). To validate this result the frequencies of retrotransposon-related sequences were compared among 454-SAM ESTs and non-normalized control ESTs from 22 maize libraries (Table S5). Consistent with the results of the microarray experiment, about 14% of 454-SAM ESTs from B73 exhibit similarity to retrotransposons, as compared with only about 1.4% of the control ESTs (see Experimental procedures). Because these control ESTs were derived from various maize tissues (Table S5), this finding suggests that retrotransposon-related transcripts are enriched in the SAM relative to not only seedlings but also to other organs at various stages of development.

To determine whether specific retrotransposon families are preferentially expressed in the SAM, a chi-squared analysis was conducted using 20 well-characterized retrotransposon families on the 454-SAM ESTs and control ESTs (library 947, 2-week-old shoots; Table S5) prepared from tissue (2-week-old shoots) quite similar to the seedlings used in our microarray analysis. A significantly larger proportion of the 454-SAM ESTs match characterized retrotransposons than do seedling ESTs (Table 5). Eight of the 20 retrotransposon families are over-represented within the 454-SAM ESTs relative to the seedling ESTs (Table 5). This finding is generally consistent with the microarray results.

The composition of nearly 290 000 454-SAM ESTs derived from the maize inbred line Mo17 (see Experimental procedures; Barbazuk *et al.*, 2007) was also analyzed. The frequencies of retrotransposon-related transcripts among the B73 and Mo17 454-SAM ESTs were compared with frequencies in existing ESTs derived from a Black Mexican Sweet (BMS) suspension culture and sperm because

Table 4 Up-regulated expressed sequence tags (ESTs) with retrotransposon-related sequences

Accession no.	*P* value	Fold change	Retrotransposon family[a]
DV942864[b]	6.4×10^{-7}	8058	Tekay
DV491560[c]	2.1×10^{-6}	5647	Ji
DV490676	1.2×10^{-6}	2494	Prem-1
DV491600	3.8×10^{-6}	1414	SDR[d]
DV489538	1.2×10^{-5}	817	Cinful
DY400775	5.3×10^{-6}	761	Ji
DV551232	1.7×10^{-5}	749	Ji
DV550068	2.9×10^{-5}	697	Grande
DV491452	2.5×10^{-6}	250	Other (centromeric)
BM336786	1.9×10^{-5}	188	Huck
DV943366	4.0×10^{-6}	178	Prem-1
DV492877	9.5×10^{-5}	107	Opie
BI359476	9.3×10^{-5}	82	Xilon
DV549339	1.4×10^{-6}	55	Prem-1
DV492067	2.3×10^{-6}	50	Xilon
BG458463	4.7×10^{-6}	50	Prem-1
BI389372	9.6×10^{-6}	48	Eninu
DN213239	6.8×10^{-7}	44	Cinful
DV495454[b]	1.4×10^{-5}	43	Cinful
DN232235	4.2×10^{-6}	38	Xilon
DY399286	3.9×10^{-5}	36	Ji
DY576450	3.5×10^{-8}	35	Cinful
DN234405	1.9×10^{-6}	31	Cinful
BI361023	2.6×10^{-5}	30	Eninu
DN206551	1.8×10^{-6}	27	Giepum
DY576322	8.0×10^{-6}	25	Dagaf
DV621178	5.4×10^{-5}	22	Opie
DN205039	6.2×10^{-6}	20	Cinful
DN204378	6.8×10^{-5}	20	Dagaf/Opie
DY542802	4.7×10^{-5}	19	Milt

The 30 of the 62 up-regulated retrotransposon-related ESTs that exhibited highest fold changes are presented.

[a]In most instances, these ESTs exhibit >85% nucleotide identity to the indicated retrotransposons over their entire lengths (data not shown).

[b]Quantitative RT-PCR analysis was performed (see legend to Figure 3).

[c] Semi-quantitative RT-PCR was performed (Table S4).

[d]SDR, statistically defined repeat homologous with a putative polyprotein sequence (*e*-value 1×10^{-10}).

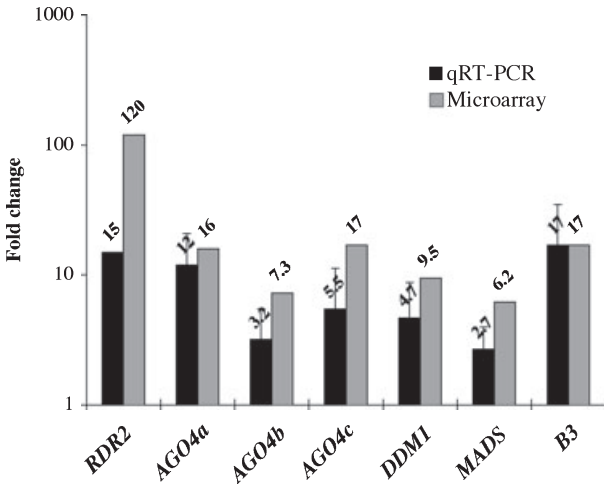

Figure 3. Quantitative RT-PCR (qRT-PCR) analyses on seven significantly up-regulated expressed sequence tags (ESTs) derived from the microarray analysis.

Fold changes [shoot apical meristem (SAM)/seedling] from qRT-PCR analyses (black bars) and the microarray analysis (gray bars) are indicated on a logarithmic scale with seven significantly up-regulated ESTs derived from the microarray analysis: *RDR2*, *AGO4a* (accession no. DV493575), *AGO4b* (accession no. DV943274), *AGO4c* (accession no. DV492375), *DDM1* (Table 3), *MADS*, and *B3*. *AGO4a*, *AGO4b*, and *AGO4c* have similar but distinct sequences (data not shown), indicating that these ESTs were derived from paralogous loci (Table 3). Means of the two biological replications (*RDR2*) and means + SD of the three biological replications (*AGO4a*, *AGO4b*, *AGO4c*, *DDM*, *MADS*, and *B3*) are shown. For Cinful and Tekay, one of the seedling samples (replication 4, Appendix S1) did not yield fluorescence above the threshold level whereas the corresponding SAM sample in the same replication did yield fluorescence above threshold level. Fold changes of the single replications for Cinful and Tekay were 7904 and 446, respectively.

retrotransposons can be expressed in tissue-cultured cells (Hirochika *et al.*, 1996) and sperm (Engel *et al.*, 2003). As shown in Table S6, the B73 and Mo17 SAM 454-ESTs contain a significantly larger proportion of retrotransposon-related sequences than do the BMS ESTs. Retrotransposon-related sequences were twice as common among the sperm ESTs as among the 454-SAM ESTs from B73 and Mo17 (Table S6). This result must, however, be considered within the context of using an *e*-value cut-off of $\leq 10^{-10}$ on short (~100 bp) 454-SAM ESTs, which could result in false-negative matches among the 454-SAM ESTs.

The frequencies of the families of retrotransposon-related transcripts vary among the 454-SAM ESTs (Tables 5 and S6). For example, among the 454-SAM ESTs from B73 the Cinful, Ji, Opie, Prem-1 and Zeon families each comprised approximately 1 to 2% of the 454-SAM ESTs, whereas ESTs classified into most of the other analyzed families comprised less than 0.2% of the 454-SAM ESTs. Despite some statistical differences this pattern is generally shared among the 454-SAM ESTs from B73 and Mo17. In contrast, there are differences in the frequencies of the analyzed families of retrotransposon-related sequences in the 454-SAM ESTs from B73 versus both the BMS and sperm ESTs (Table S6). For example, some families such as Giepum and Ruda are more abundant among the 454-SAM ESTs from B73 than the sperm ESTs, whereas the opposite pattern is observed in other families such as Doke and Xilon.

Discussion

Over 13% (about 5000) of the informative ESTs on the three microarrays were significantly up- or down-regulated in LCM-collected SAMs as compared with seedlings. The up- and the down-regulated ESTs differed in several respects (Figure 2, Tables 1 and 2). Four times more up- than down-regulated ESTs were found in the Apex ESTs derived from SAM-enriched tissues, whereas the opposite pattern was

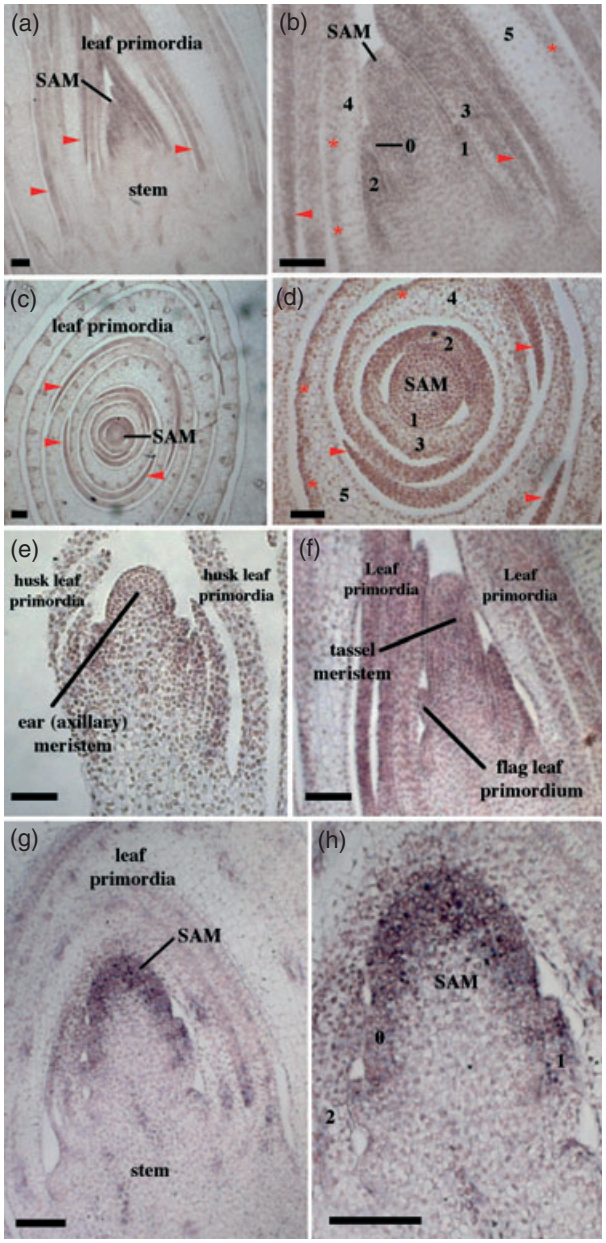

Figure 4. Transcript accumulations of maize homologs of *RDR2* and *VAP* in the shoot apical meristem (SAM), inflorescence meristems, and leaf primordia. Fourteen-day-old maize shoot apices were analyzed by *in situ* hybridization using antisense probes prepared from maize homologs of *RDR2* (a–d) and *VAP* (g, h) cDNA clones. A 21-day-old ear inflorescence apex (e) and a 28-day-old bolting tassel inflorescence (f) were also analyzed for the *RDR2* homolog. (a), (b) and (e)–(h) are images of longitudinal sections; (c) and (d) are transverse sections. (b), (d), and (h) are enlarged images of (a), (c), and (g), respectively. Leaf primordia are numbered as described in Figure 1. Red arrowheads demonstrate *RDR2* homolog transcript accumulation in leaf primordial margins; red asterisks demonstrate *RDR2* homolog mRNA accumulation in the abaxial domains of P4 (4) and P5 (5) leaf primordia. Bar: 100 μm.

observed with the ESTs derived from 2-week-old shoots that are quite similar to the seedlings used in our microarray analysis (Table 1). These data confirm that LCM-microarray analyses of the maize SAM do indeed enrich for apex-derived genes. The down-regulated ESTs had generally higher fold changes than did the up-regulated ESTs (Figure 2). Because seedlings contain more cell types than do SAMs, it is likely that the seedling transcriptome is more complex than that of the SAM. The patterns observed in Figure 2 are consistent with this relationship [i.e. if a given gene is expressed in the seedling but not in the SAM, the fold change of the transcript accumulation (seedling/SAM) is

Table 5 Percentages of expressed sequence tags (ESTs) corresponding to 20 characterized retrotransposon families for the two maize EST libraries

Retrotransposon family	454-SAM ESTs (260 736)[a]	2-week-shoot ESTs (8878)[b]
Athila	0.00	0.00
Bosohe	0.00	0.00
Cinful	1.38*	0.01
Dagaf	0.13	0.00
Diguus	0.12	0.00
Doke	0.05	0.00
Eninu	0.01	0.00
Giepum	0.32*	0.00
Grande	0.19*	0.00
Gyma	0.10	0.05
Huck	0.15	0.02
Ji	1.79*	0.02
Milt	0.09	0.00
Opie	0.93*	0.01
Prem-1	1.19*	0.10
Prem-2	0.06	0.00
Ruda	0.12	0.00
Tekay	0.13	0.00
Xilon	0.45*	0.00
Zeon	1.90*	0.08
Other[c]	0.23*	0.01
Total	9.35*[d]	0.30

BLASTN searches were performed for two maize EST libraries (see Experimental procedures). A chi-squared homogeneity test was performed to identify retrotransposon families that were present at significantly ($P < 0.001$) higher proportions among the shoot apical meristem (SAM) ESTs than among shoot ESTs. Such families are marked by an asterisk (*). The total number of ESTs from each library used in this analysis is presented in parentheses. Retrotransposon families that were up-regulated in the microarray experiment (Tables 2 and 4) are italicized. Although not presented in Table 4, Gyma (DN224485, 2.1-fold) and Zeon (DY399271, 7.6-fold; DN210516, 4.2-fold; DN213990, 3.2-fold) were also significantly ($P < 0.0001$) up-regulated in the SAM.

[a]454-SAM ESTs were derived from laser capture microdissection-collected B73 SAMs (see Experimental procedures).

[b]2-week-shoot ESTs were derived from 2-week-old shoots of maize (Table S5).

[c]Includes other characterized but minor retrotransposon families detected among the 454-SAM ESTs, e.g. Hopscotch.

[d]Overall, about 14% of the 454-SAM ESTs exhibit similarity to retrotransposons. The total shown in this table includes only those 454-SAM ESTs that exhibit similarity to the characterized retrotransposons listed here.

generally very high]. Genes predicted to function in chromatin regulation, cell division and DNA repair were over-represented among the up-regulated SAM transcripts (Table 2). This is reasonable given that much of the SAM comprises dividing cells undergoing active DNA replication. On the other hand the photosynthesis-related category was over-represented among the down-regulated ESTs (Table 2), which is also expected because the SAM is heterotrophic (Fleming, 2006). These LCM-coupled microarray results were further validated by several independent types of experiments, including consistency with published expression patterns (Table S3), semi-qRT-PCR (Table S4), qRT-PCR (Figure 3), and *in situ* hybridization (Figure 4).

Sequencing cDNAs complemented our analysis of SAM-expressed genes. The Apex ESTs, which we prepared from manually collected maize apices, were spotted on our arrays (Table S1). This resulted in the identification of 312 up-regulated 'Apex-unique' ESTs (Table 1). In addition, over 260 000 454-SAM ESTs derived from the LCM-collected B73 SAMs aligned to about 25 800 genomic loci in maize (Emrich *et al.*, 2007). Hence, this study provides evidence that about 50% of the 50 000 maize genes (Fu *et al.*, 2005; Haberer *et al.*, 2005) are expressed in the SAM.

The microarray analysis identified many regulatory genes that were up-regulated in the SAM. These up-regulated genes included those encoding transcription factors, chromatin remodeling factors, and components of the gene-silencing machinery (Table 2). The stem cell functions of the SAM and leaf development are regulated by a variety of transcription factors (Hay *et al.*, 2004). Consistent with this our microarray analysis annotated 173 up-regulated ESTs as being transcription factors (Table 2), many of which are uncharacterized. The up-regulation of two of them, *MADS* and *B3*, was confirmed via qRT-PCR (Figure 3). *MADS* (DY401890) is identical to the maize putative MADS-domain transcription factor gene, *m22* (AJ430636). Members of MADS-box family are diverse and are involved predominantly in developmental processes. The Arabidopsis genome contains 107 MADS-box family genes (Parenicova *et al.*, 2003). *AGL19*, the most similar Arabidopsis homolog of *m22* is expressed mainly in roots (Alvarez-Buylla *et al.*, 2000; Parenicova *et al.*, 2003). Our microarray results, however, indicate that in maize *m22* is expressed in the SAM, whereas to our knowledge there has been no report that *m22* is expressed in maize roots. *B3* (DN214788) exhibits similarity to Arabidopsis transcription factor genes encoding auxin response factor 36 and VRN1 (reduced vernalization response 1) (Levy *et al.*, 2002). *B3* is one of the 312 up-regulated ESTs that were 'Apex-unique' (Table 1), suggesting the possibility that *B3* is a specific regulator of SAM function.

Chromatin remodeling factors regulate gene expressions in the SAM (Guyomarc'h *et al.*, 2005; Kwon *et al.*, 2005; Phelps-Durr *et al.*, 2005). Ninety-one up-regulated ESTs were annotated as chromatin remodeling in our microarray analysis (Table 2).

Several up-regulated ESTs exhibited similarities to genes involved in RdDM and heterochromatin formation (Table 3); the up-regulation of five such genes was validated via qRT-PCR (Figure 3). Expression of the *RDR2* homolog was analyzed via *in situ* hybridization and transcripts were detected not only in the SAM *per se*, but also throughout P0 to P2 leaf primordia and in inflorescence shoot meristems (Figure 4a–f). In older leaf primordia, *RDR2* expression becomes restricted to those cells that are not fully differentiated and are actively dividing, such as those at the leaf margins. This expression pattern suggests a requirement for RdDM in mitotic tissues, and may reflect a role for RdDM in the maintenance of cytosine methylation at asymmetric CpNpN sites (Bender, 2004; Chan *et al.*, 2005). Cytosine methylation at symmetrical sites (CpG and CpNpG) can be maintained following DNA replication through the activity of DNA methyltransferases such as MET1 and CHROMOMETHYLASE3 (Bender, 2004; Chan *et al.*, 2005). Asymmetric cytosine methylation patterns cannot, however, be maintained by these DNA methyltransferase activities, but must instead be re-established *de novo* following each round of replication. In the absence of RDR2 or other components of the RdDM pathway, repeat-associated methylation would be lost progressively from dividing cells. This hypothesis is consistent with recent findings that the *mop1* gene, which is involved in the heritable epigenetic phenomena of paramutation (Alleman *et al.*, 2006) and transposon silencing (Woodhouse *et al.*, 2006), encodes the specific maize *RDR2* homolog analyzed in this study.

The expression of another up-regulated EST (DV622566), a maize *VAP* homolog, was analyzed via *in situ* hybridization (Figure 4g,h). Transcripts of the *VAP* homolog were localized in an unusual pattern at the summit of the SAM. Vesicle-associated membrane protein-associated proteins are type II integral membrane proteins localized in the endoplasmic reticulum, and are proposed to function in the secretory pathways of animals and yeast during vesicular membrane trafficking (Vedrenne and Hauri, 2006). Previous analyses revealed that intercellular trafficking of KNOTTED1 (Jackson *et al.*, 1994; Kim *et al.*, 2002) and the vesicular cycling of the PIN family of auxin efflux proteins are required for normal shoot development (Benkova *et al.*, 2003; Geldner *et al.*, 2003; Reinhardt *et al.*, 2003), and demonstrate the importance of vesicular trafficking during SAM function.

Among the differentially regulated genes were 62 that exhibited similarity to retrotransposons (Tables 2 and 4). The up-regulation of three of these retrotransposon-related ESTs was confirmed via semi-qRT-PCR (Table S4) or qRT-PCR (see legend to Figure 3). The finding that 14% of the 454-SAM ESTs were retrotransposon-related (Table 5) provides further evidence that retrotransposons are transcribed and

accumulate in the SAM. Retrotransposon-related sequences are also over-represented among ESTs from maize sperm (Engel *et al.*, 2003) and tissue-cultured cells of maize (Table S6) and Arabidopsis (Pischke *et al.*, 2006). In contrast, retrotransposon-related sequences are not transcribed to high levels in most other maize tissues (Meyers *et al.*, 2001). Recent evidence suggests that suppression of retrotransposon transcription is mediated by RdDM function during heterochromatin formation (Bender, 2004; Chan *et al.*, 2005). Hence, the finding that several ESTs that are highly similar to genes involved in RdDM and heterochromatin formation, and whose Arabidopsis homologs are known to be involved in retrotransposon silencing (Hirochika *et al.*, 2000; Lippman *et al.*, 2004; Lippman *et al.*, 2003; Xie *et al.*, 2004; Zilberman *et al.*, 2003), are up-regulated in the SAM (Figure 3 and Table 3) is somewhat paradoxical.

To explain the curious up-regulation of both retrotransposon-related transcripts and genes involved in silencing retrotransposons, we hypothesize that in the SAM (and habituated tissue culture cells) 'something' triggers the transcription of retrotransposons and in response the gene-silencing machinery is activated to reduce genome-damaging retrotranspositions. Recently a novel class of retrotransposon-derived siRNAs of 21 to 22 mers was identified in maize plants that are homozygous for a mutant allele of the *RDR2* homolog, *mop1* (B. Meyers, P. Green, and V. Chandler, personal communication). This is consistent with the presence of an RDR2-independent system to silence retrotransposons in maize. The up-regulation of retrotransposon-related transcripts in the maize SAM could therefore potentially be due to the down-regulation of this RDR2-independent system in the SAM.

The activation of retrotransposon-related sequences might be an evolutionarily conserved trait of these 'selfish DNA sequences', i.e. sequences that replicate in mitotically active, meristematic cells would have increased chances of being selected and amplified in subsequent generations. How might the initial activation of retrotransposon-related sequences occur? Transcripts from the various families of retrotransposon-related sequences do not accumulate equally in the SAM (Tables 5 and S6). Even though Huck is one of the most abundant elements in the genome (Meyers *et al.*, 2001), it is relatively less abundant among the 454-SAM ESTs from B73 and Mo17 (Tables 5 and S6). This suggests that the transcription of retrotransposons in the maize SAM may be regulated in a family- or element-specific manner, rather than simply being the result of a genome-wide activation of retrotransposons. This could arise via the action of transcription factors specific to (or greatly enriched in) the SAM. Among the significantly up-regulated ESTs in our microarray experiment were 173 annotated transcription factors (Table 2), many of which do not yet have defined targets (e.g. *MADS* and *B3*; Figure 3). Alternatively, the transcriptional activity of a retrotransposon could depend on its chromosomal location. According to this view, retrotransposon-related transcripts may be generated selectively by isolated elements dispersed throughout euchromatic regions of the genome, even though the retrotransposons arrays characteristic for most of the maize genome are silenced throughout plant development through the formation of higher-order heterochromatin.

McClintock termed the DNA transposons she studied 'controlling elements' based on her observation that they could control gene expression (McClintock, 1951). Subsequently, other transposons have been shown to be able to regulate the expression of nearby genes (Martienssen *et al.*, 1990). Even so, except for a few exceptions (Pardue and DeBaryshe, 2003; Zhong *et al.*, 2002), there is little direct evidence for roles of retrotransposons in normal development. Hence, although it is possible that the expression of retrotransposons in the SAM does not have functional significance, here we consider the possibility that the transcription of both retrotransposons and their silencing machinery may contribute to stem cell functions. The SAM, sperm and tissue-cultured cells in which retrotransposon-related sequences are transcribed at high levels are all pluripotent. Similarly, totipotent mouse oocytes and two-cell embryos also exhibit high levels of retrotransposon-related transcription (Peaston *et al.*, 2004).

We hypothesize that in the SAM, retrotransposon-related transcripts serve as sources of siRNAs that target for silencing genes that regulate the maintenance of stem cell identity and differentiation. This hypothesis is based on the observations that genes involved in RdDM and heterochromatin formation are up-regulated in the SAM (Table 3 and Figure 3) and that at least some maize genes contain pieces of retrotransposon-related sequences in their 3′ untranslated regions (UTRs) (Appendix S1). Such genes could potentially be transcriptionally silenced via interactions with the retrotransposon-derived siRNAs.

Experimental procedures

Plant materials and growth conditions

The maize (*Zea mays* L.) inbreds B73 and Mo17 were maintained by self-pollination. Kernels were planted about 2 cm deep in plastic pots (8.5 cm × 8.5 cm wide at the top and 7.5 cm deep) filled with SB 300 Universal Mix (Sun Gro Horticulture). Pots were placed in an environmental control room (PGW-40, Percival Scientific, http://www.percival-scientific.com/). The light intensity at the surface of the growth medium was kept between 650 to 860 μmol m^{-2} sec^{-1} as measured with a quantum meter (model QMSW, Apogee Instruments, http://www.apogeeinstruments.com/). Temperature and light cycles were set at 25°C with 15-h light conditions and at 20°C with 9-h dark conditions. Seedlings were watered as needed with a solution containing 0.7 mM calcium nitrate. The SAMs and seedlings were harvested 14 days after planting.

Preparation of paraffin sections

Paraffin-embedded tissues were prepared as described by Kerk *et al.* (2003) with significant modifications. Details are provided in Appendix S1.

Collecting the maize SAM tissue with LCM

The laser microdissection and pressure catapulting (LMPC) technique, one of several LCM techniques, was used to collect maize SAMs. The PALM MicroBeam System (115V Z, P.A.L.M. Microlaser Technologies, http://www.palm-microlaser.com/) was used for LMPC. Tissue sections were deparaffinized in 100% xylene. Each SAM was divided into 10 to 15 longitudinal sections (10 μm thick), each of which was collected via LMPC except for one to two sections at each edge of the SAM, which typically had ambiguous morphology.

RNA extraction

The SAMs were collected via LCM into the extraction buffer of the PicoPure RNA Isolation Kit (Arcturus, http://www.arcturus.com/) and RNA extracted according to the manual. The RNA samples were treated with RNase-free DNase I (Stratagene, http://www.stratagene.com/) while on the column using the DNase incubation buffer provided with the PALM RNA extraction kit (P.A.L.M. Microlaser Technologies). Appendix S1 describes RNA extraction to determine RNA yields from maize SAMs and RNA extraction from maize seedlings.

T7 RNA polymerase-based RNA amplification

T7 RNA polymerase-based (T7-based) RNA amplification was performed according to the method of Nakazono *et al.* (2003) with slight modifications. Approximately 10 ng of RNA was used per amplification (Table S2).

ESTs included on the microarrays

Amplicons from cDNA clones were prepared and spotted on microarrays as described previously (Nakazono *et al.*, 2003; Swanson-Wagner *et al.*, 2006). Details regarding the three microarrays used in this study [SAM1.0 (GPL2557), SAM2.0 (GPL2572), and SAM3.0 (GPL3538)] are available at http://www.plantgenomics.iastate.edu/maizechip/. In total, these arrays contain 37 660 informative EST spots. Approximately 30% of these spots were derived from the 'Apex ESTs' generated as part of the current study (Table S1). Because many genes (Emrich *et al.*, 2007), e.g. *kn1* (Jackson *et al.*, 1994), are expressed in both vegetative SAMs and reproductive meristems, ESTs derived from maize tissues that are enriched with reproductive meristems were also included on the microarrays. Approximately 6700 ESTs (about 18% of the total informative spots) were derived from 0.2 cm ears (library 3529, 0.2 cm ear tissue) that included inflorescence meristems and spikelet pair meristems. Expressed sequence tags derived from slightly larger immature ears (0.5–2 cm ears, libraries 606 and 1091, immature ear tissue) and tassel primordia (0.1–0.3 cm, library 946, tassel primordia; 0.1–2.5 cm, library 618, inbred tassel) were also included, which together comprise about 10% of the total. Immature ears that are 0.5–2 cm long include developed spikelet meristems, and immature floral organs such as carpels. Tassel primordia that are 0.1–0.3 cm long include branch meristems, inflorescence meristems, and spikelet pair meristems. Tassel primordia that are 0.3–2.5 cm long include developed spikelet meristems and immature floral organs, as well as branches with spikelet pair and spikelet meristems.

Synthesis of fluorescent probes for microarray hybridization

Two micrograms of aRNA were labeled according to Nakazono *et al.* (2003) with slight modifications. To remove dye-specific effects in the statistical analyses, Cy dyes were swapped between the RNA samples with odd and even numbers (Table S2). Microarray hybridizations were performed according to Swanson-Wagner *et al.* (2006).

Microarray analysis

Each of the SAM1.0 and SAM2.0 arrays was scanned seven times with a ScanArray 5000 (Packard BioScience, now PerkinElmer, http://www.perkinelmer.com/) according to Swanson-Wagner *et al.* (2006) except that three scan sets were selected (low, medium, and high signal intensity) from each of the 12 slides used in the experiment. Each of the SAM3.0 arrays was scanned nine times with a Pro Scan Array HT (PerkinElmer) with increasing laser power and fixed photomultiplier tube gain settings. As with the SAM1.0 and SAM2.0 arrays three data sets were selected for analysis from each SAM3.0 array. By analyzing data collected using multiple scan settings we expected to detect more differentially expressed genes (Skibbe *et al.*, 2006).

Prior to statistical analyses, 4628 'empty', 'bad-PCR' and other non-informative spots were removed from the data set. Details about data normalization, data centering and statistical analysis are provided in Appendix S1. On a spot-by-spot basis, the scan with the smallest *P* value was selected for subsequent analyses. Following statistical analyses, an additional 6608 spots were removed from the data set because of concerns regarding the quality of the associated DNA sequences; 384 control spots that intentionally contained exogenous DNA were also removed. As a result, this study reports the gene expression patterns of 37 660 'informative' spots from the three microarrays. Microarray data have been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE6267.

In situ *hybridization*

In situ hybridizations of maize tissues were performed by the method of Jackson (1991) with changes as described in Jackson *et al.* (1994) and in Juarez *et al.* (2004a). Plasmid DNA of the EST clone for the maize *RDR2* homolog (accession no. CB816774, insert size ~500 bp) and *VAP* homolog (accession no. DV622566, insert size ~1.25 kb) were digested with *Eco*RI and anti-sense RNA probes synthesized. Hybridizations were performed on sections of 14-day-old B73 seedling apices. For the maize *RDR2* homolog, the probe was also hybridized to sections of inflorescence apices derived from 21-day-old seedlings (the ear inflorescence) and 28-day-old (the bolting tassel inflorescence) seedlings of B73.

454 sequencing

Double-stranded cDNA was synthesized from 20 μg of the aRNA samples from SAMs (a mixture of replications 2 and 4; Table S2)

using methods for first and second strand synthesis of the second round amplification in the T7-based RNA amplification described above with minor modifications. Fifteen micrograms of cDNA was recovered and used for sequencing at 454 Life Sciences (Margulies *et al.*, 2005). The resulting sequence data were then processed using Lucy (Chou and Holmes, 2001) to remove low-quality sequences and to trim low-quality polyA/T stretches. After removing a small amount of *Escherichia coli* contamination using SeqClean (http://www.tigr.org/tdb/tgi/software) 260 736 high-quality sequences (454-SAM ESTs from B73, 101-bp average length without polyA/T tails) were submitted to GenBank (Emrich *et al.*, 2007). Similarly, 287 917 ESTs from LCM-collected Mo17 SAMs (100-bp average length without polyA/T tails) were submitted to GenBank. These ESTs were prepared and processed in the same manner as were the B73 454-ESTs (Emrich *et al.*, 2007).

BLAST searches for chi-squared analyses

Procedures for constructing the retrotransposon database (1679 sequences) and collecting EST data sets from GenBank are provided in Appendix S1. The 260 736 454-SAM ESTs from B73 and the 65 215 control ESTs (Table S5) were annotated via BLASTN to the retrotransposon database (using $>1 \times 10^{-10}$ as the cut-off). Because duplicates were not removed, this approach is a conservative estimate of retrotransposon expression in the tissues analyzed. Equality of retrotransposon frequencies in specific families between different EST collections was tested using a chi-squared test based on the best BLASTN match (e-value $\leq 1 \times 10^{-10}$) to 1339 characterized retrotransposon sequences and a P value cut-off of 0.001.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Quality check of amplified RNA (aRNA) samples.

Table S1. Clustering of Apex expressed sequence tags (ESTs) and other maize ESTs.

Table S2. RNA samples for microarray experiments.

Table S3. Shoot apical meristem control genes.

Table S4. Semi-quantitative RT-PCR analyses of two significant expressed sequence tags.

Table S5. Twenty-five maize cDNA libraries used for BLAST searches for chi-squared analyses.

Table S6. Percentages of expressed sequence tags (ESTs) corresponding to 20 characterized retrotransposon families for six maize EST libraries.

Appendix S1. Detailed experimental procedures that are not presented in the printed version of this article.

This material is available as part of the online article from http://www.blackwell-synergy.com

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