Transposable elements (TEs) are ubiquitous members of all higher eukaryotic genomes and compose the majority of DNA present in the maize genome (Schnable et al., 2009). These mobile DNAs are eukaryotic genomes and remain poorly defined even in well-studied genomes such as maize. We treated maize tissue culture with the demethylating agent 5-aza-2-deoxycytidine and examined long-term tissue culture lines to discover silenced TEs that have the potential to induce heritable genetic variation. Through these screens we have identified a novel low copy number hAT transposon, Tissue Culture Up-Regulated (TCUP), which is transcribed at high levels in long-term maize black Mexican sweet (BMS) tissue culture and is transcribed in response to treatment with 5-aza-2-deoxycytidine. Analysis of the TIGR Maize Gene Index revealed that this element is the most frequently represented EST from the BMS cell culture library and is not represented in other tissue libraries, which is the basis for its name. A full-length sequence was assembled in inbred B73 that contains the putative functional motifs required for autonomous movement of a hAT transposon. Transposon display detected novel TCUP insertions in two long-term tissue-cultured cell lines of the genotype Hi-II × B and BMS. This research implicates TCUP as a transposon that is capable of reactivation and which may also be particularly sensitive to the stress of the tissue culture environment. Our findings are consistent with the hypothesis that epigenetic alterations potentiate genomic responses to stress during clonal propagation of plants. 

Keywords: transposable element, tissue culture, DNA methylation, genome stress, epigenetics, somaclonal variation

INTRODUCTION

Transposable elements (TEs) are ubiquitous members of all higher eukaryotic genomes and compose the majority of DNA present in the maize genome (Schnable et al., 2009). These mobile DNAs are critical elements in the creation of genetic variation that can drive the reorganization and evolution of genomes. Even though these repetitive elements make up an extensive proportion of higher eukaryotic genomes, they are normally maintained in a transcriptionally silent and immobile state (Chomet et al., 1987; Lippman et al., 2003, 2004; Dooner and Weil, 2007). TEs are known to become activated from their dormant state by both biotic (Johns et al., 1985; Grandbastien et al., 1997; Kashkush et al., 2003; Xu et al., 2004) and abiotic stresses (Neuffer, 1966; Carpenter et al., 1987; Peschke et al., 1987; Kalendar et al., 2000; Kimura et al., 2001; Jiang et al., 2003).

The transcription of TEs near or within genic regions can change the spatial and temporal expression of genes leading to altered phenotypes by both genetic and epigenetic mechanisms (Morgan et al., 1999; Kashkush et al., 2003). They may also be mutagenic when mobilized and transposes or near genes. The mobilization of all types of transposons requires transcription from autonomous elements. Activation of dormant TEs may be a contributor to the phenomenon of somaclonal variation. Somaclonal variation is defined as genetic or phenotypic variation among clonally propagated plants derived from a single donor clone and results in a wide range of phenotypes displayed in the progenies of regenerated plants (Kaeppler et al., 2000).

Somaclonal variation is a general loss of DNA methylation in heterochromatic regions, but specific elements become hypomethylated and gain H3Kme2 in both heterochromatic and euchromatic chromosome locations

Transposon-induced activation of silenced TEs does not affect all elements equally, with some elements predictably activated by the culture process (Hirochika et al., 1996; Takeda et al., 1999), and most others remaining quiescent. For example, silenced Mutator elements are not reactivated through tissue culture and slightly active elements can become methylated and silenced in tissue-cultured lines (Planckert and Walbot, 1989). Other TEs, such as Tto1, are predictably activated during tissue culture. Tto1 contains cis regulatory sequences sensitive to the auxins used in tissue culture, providing an explanation for the sensitivity of this element to culture stress (Takeda et al., 1999).

The cell culture transcriptome of maize is enriched with TE ESTs compared with other organ tissue, but not all TEs are equally transcribed. The hAT transposon Ma is transcribed while the related element Ac is not, highlighting the differential response of TE to the tissue culture process (Vicient, 2010). The maize miniature inverted repeat TEs (MITE) ZmTPApong is transcriptionally activated in cell culture and mobilized in the regenerated progeny (Barret et al., 2006) and the MITE related P instability factor (PIF) is enriched in cell culture compared to other tissues (Vicient, 2010). The activation of TEs has been associated with a general loss of DNA methylation in heterochromatic regions, but specific elements become hypomethylated and gain H3Kme2 in both heterochromatic and euchromatic chromosome locations
The unique sensitivity of different TEs to specific stresses underlies the types and frequency of genetic variation induced in specific environments. The goal of this research was to characterize a novel maize class 2 hAT transposable element that was first identified based on high expression specifically in black Mexican sweet (BMS) long-term cell culture. Based on its initial in silico identification indicating EST evidence of high expression in BMS and no expression in other tissues, we named the element Tissue Culture Up-Regulated (TCUP). A subsequent pharmacological analysis using the DNA methylation inhibitor, 5-aza-2-deoxycytidine, was used to evaluate the effect of DNA methylation on TCUP transcription in newly initiated cultures. The mobility of TCUP was also evaluated in a series of long-term Hi-II A × B tissue culture lines.

MATERIALS AND METHODS

CALLUS LINES

The BMS cell line was initiated in the 1970s at the University of Minnesota and was most recently acquired for our studies from Charles Armstrong at Monsanto in 2001. Independent callus lines were created from individual Hi-II A × B embryos harvested 12 days after pollination. Callus lines were maintained on N6 media supplemented with 1.5 mg/l of 2,4-dichlorophenoxyacetic acid (Armstrong, 1994). The embryogenic cell cultures were transferred to fresh media monthly.

PLANT MATERIAL

The inbred stocks obtained from the Maize Genetics Cooperation Stock Center were BMS (Accession: B542B), Hi-II A (Accession: T0940A), and Hi-II B (Accession: T09040B) (Armstrong et al., 1991). All seeds were bulked and maintained using sib crosses at the Maize Genetics Cooperation Stock Center were BMS (Accession: B542B), Hi-II A (Accession: T0940A), and Hi-II B [Accession: T09040B] (Armstrong et al., 2001). Independent callus lines were created from individual Hi-II A × B embryos harvested 12 days after pollination. Callus lines were maintained on N6 media supplemented with 1.5 mg/l of 2,4-dichlorophenoxyacetic acid (Armstrong, 1994). The embryogenic cell cultures were transferred to fresh media monthly.

SUSPENSION CULTURE TREATMENTS

Each culture line was initiated using 1.5 g of Hi-II A × B type II embryogenic callus broken into small clumps. The culture lines were each split into two flasks prior to initiation of the experiment. One flask within each of the three cell lines was treated with 25 µM 5-aza-2-deoxycytidine, and the other flask was used as a non-treated control. Liquid N6 medium was replaced with either treated or untreated medium every 3 days for 9 days to ensure an adequate treatment length.

GENOMIC DNA ISOLATIONS FROM PLANT TISSUE

Genomic DNA was isolated from plant tissue using the CTAB method (Saghai-Maroof et al., 1984). The DNA was suspended in LTE (1 mM Tris–HCl pH 8.0, 0.1 mM EDTA pH 8.0). DNA was extracted from callus using the Plant DNAzol reagent (Invitrogen catalog # 10978-021).

PCR AMPLIFICATION OF GENOMIC DNA

PCR reactions contained the following components: 1× Taq DNA polymerase buffer, 2.0 mM MgCl₂, 200 µM dNTPs, 0.6 µM each primer, 1 U Taq DNA polymerase, 100 ng of genomic DNA, and sterile distilled deionized water to a volume of 25 µl. Cycling parameters were generally as follows: 94˚C 2 min, 30–35× (94˚C for 30 s, 58˚C for 45 s, 72˚C 1 min per kilobase of amplicon) 72˚C for 7 min. TCUP 5′ probe sequence corresponding to 9–875 bp of accession DQ324364.1 was amplified using primers TCUP5′F GCCAAATGCGACTAACAGCGC and TCUP5′R GAGGAGATACAGTGGCCAG. The TCUP internal probe sequence corresponding to 2203–3439 bp was amplified using primers InternalF GCTGGTGTGTTGCTGATTATG and InternalR CGTCGATTGATCTGGCCAGT. The TCUP 3′ probe sequence corresponding to 3313–4127 bp was amplified using primers TCUP3′F GGTGGCATCAGCAAAACTCA, TCUP3′R TATA-GATGGCCACCGGCGCAGGCAG. Reamplification of the excised and sequenced novel band from transposon display was performed using h6_band1 CAGGCGCCGAACTTGACATAG and display TCUP3′-1 ACTGGTATGCGGCTGGTGCCG.

DNA SEQUENCING

Sequencing reactions consisted of 1 µl of BigDye Terminator mix version 3.1 (Applied Biosystems), 1.5 µl of BigDye buffer version 3.1 (Applied Biosystems catalog # 4336697), 1 µM of primer, and 0.2 µg of plasmid DNA in a final reaction volume of 10 µl. The cycle parameters were 95˚C for 3 min; 35× (96˚C for 10 s; 55˚C for 15 s; 60˚C for 3 min) followed by 72˚C for 7 min. Sequence was determined at the University of Wisconsin Biotech Center DNA Sequencing facility.

DNA GEL BLOT ANALYSIS

Genomic DNA (5 µg) was digested with 20 U of restriction enzyme under manufacturer’s specified conditions for 16 h. Digested genomic DNA was electrophoresed through 1% TBE gels and transferred using the neutral transfer protocol supplied with Hybond N+ membranes (Amersham catalog # rpm203b) according to manufacturer’s instructions. After transfer, blots were UV crosslinked and baked for 2 h at 80˚C. Prehybridization was performed with hybridization buffer (5× SSPE, 5× Denhart’s, 1% SDS, 100 µg/ml sheared salmon sperm DNA) for 2–5 h at 65˚C. Hybridizations were performed in hybridization buffer at 65˚C for 16 h with denatured [α-32P] dCTP (Perkin Elmer catalog # BLU513H) labeled probes. After hybridization, blots were stringently washed with 0.2× SSC, 0.2% SDS three to four times at 65˚C for 20 min each.

PROBE GENERATION AND LABELING FOR DNA GEL BLOT ANALYSIS

Segments of TCUP were PCR amplified from B73 genomic DNA, cloned using the pGEM™-T Easy Vector System II kit (Promega catalog # A1380), and confirmed by sequencing. The clones used as probes for DNA gel blots are as follows: TCUP5′ bp 9–875; TCUP internal bp 2203–3439; TCUP 3′ bp 3313–4127. Random labeling was performed with 25 ng of purified probe using the Prime-a-Gene 5× labeling buffer (Promega catalog # U1151).

INFORMATIC AND PHYLOGENETIC ANALYSIS

The TCUP sequence (GenBank Accession DQ324364) was completed by manual assembly of PCR sequences and sequence from trace archives for BAC clone ZMMBBc0310A01
(GenBank AC148160). The translated protein sequence of TCUP was analyzed for known domains using the BLAST-based NCBI conserved domain database (Marchler-Bauer and Bryant, 2004; Marchler-Bauer et al., 2005). The default settings were used for all searches. The position of this sequence in version 2 of the B73 reference genome (AGPv2; http://ftp.maizesequence.org/current/assembly/; Schnable et al., 2009) was determined in February 2011 on chromosome 7 corresponding to contig AC195167 derived from BAC clone ZMMBB80310A01. The location of the insertion was confirmed by identification of the target site duplication (TSD) sequence AAGCAAAAG.

Protein sequences were aligned using ClustalX (Thompson et al., 1997). The phylogenetic analysis was performed on Clustal aligned transposase protein sequences according to Xu and Dooner (2005). All transposases used in the alignment were derived from putative full-length elements except for the sorghum sequence translated from TC93360 which appears to be a partial transposase. The phylogenetic method was created using the MEGA3 program (Kumar et al., 2004) using the neighbor joining method by performing 1000 bootstraps. The pairwise deletion method was selected to handle gaps in the alignments.

The accession numbers used in alignment are as follows: DQ324364 TCUP Zea mays, S13518 snapdragonTam3, P08770 Maize Ac ORF, AA32822.1 Maize Mx, AAB60236.1 House Fly Hermes, AAD24567.1 Arabidopsis thaliana Tag2, BAA36225.1 Ipomoea purpureaTip100, CAA66182.1 N. tabacum Slide, AAL39203.1 Bactrocera dorsalis Hopper, CAA93759.1 Toxopneustis inflatum Restless, P12258 D. melanogaster Hobo, BAB01787.1 A. thaliana Tip100, TC93360 Sorghum bicolor TCUP-like, BAD93710.1 Oryza sativa TCUP-like, P002409 O. sativa TCUP-like S, AP002071 O. sativa TCUP-like L, 201344A P. glaucum Pat2, CABB8266.1 A. thaliana, NP191811.1 A. thaliana, AAL31650.1 O. sativa, BAA96580.1 O. sativa, AAM97760.1 O. sativa, NP20480.1 O. sativa, NP118098.2 A. thaliana, AAB28302.1 C. parasitica Crypt, BAA22244 F. oxysporum Tfo1, AAA64851 L. cuprina Hermit, AAD03082 B. tryoni Homer, XP309253.2 A. gambiae, NP192758.1 A. thaliana.

**RNA EXTRACTIONS**

RNA was extracted using the TRIZOL reagent (Invitrogen catalog # 15596-026) following the manufacturer’s protocol with optional spin steps included for plant tissue. The callus RNA extraction protocol had the following modifications: callus RNA was extracted using an extra partitioning with chloroform to reduce phytogel contamination in the RNA pellet.

**REVERSE TRANSCRIPTASE-PCR**

Total RNA (5 μg) was DNase treated [1× RQ1 RNase-Free DNase 10× buffer, 2 μg RQ1 RNase-Free DNase (Promega catalog # M6101)] for 20 min at 37°C. The cDNA was primed with Oligo (dT)15 (Promega catalog # C1101). Reverse transcription was carried out using 1× M-MLV reaction buffer, 0.5 mM dNTPs (Promega catalog # U1330), 200 U M-MLV reverse transcriptase (Promega catalog # M1701), 20 U RNAsin (Promega catalog # N2511). The reaction was incubated at 42°C for 2 h and then heated to 70°C for 10 min to stop the reaction.

PCR was performed using the following conditions [1× Tth Buffer (Epitome catalog # TTH72250), 2 mM MgCl2, 0.4 μM primers, 3× Masteramp enhancer, 1 U Tth DNA polymerase] on 3–6 μl of cDNA and cycled 25 times (94°C for 35 s, 58°C for 45 s, 72°C for 60 s). The 2.15-kb fragment was amplified using primers TCUP3′RACE2 AAGATGAAACTTGAGTCGACTGG and TCUP3′rev.1 ACCACAGAAGCTCAATCCTCCTCCCT.

**5′ AND 3′ RAPID AMPLIFICATION OF cDNA ENDS**

Rapid amplification of cDNA ends (RACE) was performed using materials and the protocol from the BD SMART™RACE cDNA Amplification Kit (BD Biosciences catalog # 634914). The PCR amplicons from nested PCR were isolated using the QiAquick Gel Extraction Kit (Qiagen catalog # 28704) and cloned using the pGEM®-T Easy Vector System II kit (Promega catalog # A1380). The TCUP specific primers used for 5′ RACE were 5′ TCUP RACE GTGTCAAGGAACCTCATGCGG, TCUP2R5′TCACATTACCTCATGGCCAC, TCUP5′RACE1 AAGATGGAAGGGACACCCGGTTCA, TCUP5′F-CL307541 CATGTCTCTGTGGGTGGTTTG, and TCUP5′RACE3 CCTCTGATGGTGACTTACGACATGTC. The TCUP specific primers used for 3′ RACE were TCUPRACE3 GTGATGTGTGTGGATGACCGACTGG, TCUP3′RACE TCCTAGTCTTGCTCTCTATTAGCT, TCUP3′RACE2 AAGATGGAACCTTGAGATCGACTGG, and TCUP3′RACE3 CCTACTCGTTGAGGACCG.

**TRANSPOSON DISPLAY**

Transposon display was performed following the protocol described by Casa et al. (2004) using genomic DNA isolated from long-term callus lines and inbred plant DNA. The TCUP specific primers used for transposon display were disTLE3′-1 ACTTGATAGTGGCGTCTGG, disTCUP3′-1 GGTGACCGACCTCATGCGG, disTCUP5′-1 GTGATGGAAGGGACACCGGGTT, disTCUP5′-1nest GTGATGGAAGGGACACCGGGTT, and disTCUP5′-1nest GTGATGGAAGGGACACCGGGTT.

**RESULTS**

**IDENTIFICATION AND CHARACTERIZATION OF TCUP**

Tissue Culture Up-Regulated was first identified as TC249207 by evaluation of EST abundance in BMS suspension cultures using the TIGR gene index. This was the most abundant transcript from the B73 and there was no evidence of expression in any other tissue based on this gene index. This observation led to us to name the element as TCUP. Expression of TCUP in BMS, newly initiated cell cultures, seedling leaf, seedling root, endosperm and developing endosperm was expressed by RT-PCR and TCUP transcription was not detected in seedling, leaf, root, endosperm, and newly initiated untreated cultures. These evaluations supported that at least one member of the TCUP family is actively transcribed in BMS callus cultures, and that TCUP is stably quiescent in other plant tissues as evaluated by EST and RT-PCR.

A full-length TCUP sequence (GenBank accession DQ324364) was assembled and confirmed using informatics and targeted sequencing (Figure 1). The sequence is 4132 bp in length and contains a single intronless 738 amino acid open reading frame.
The AGP version 2 position of this sequence is on chromosome 7, but bridges several contigs AC195167.2-contig69, AC195167.2-contig72, and AC195167.2-contig36 within the unordered BAC clone ZMMBBc0310A01. The assembly of these contigs does not produce a contiguous sequence suggesting that the assemblies utilized for AGP version 2 of this BAC clone may not be correct. An alternate assembly for BAC clone ZMMBBc0310A01 was identified (AC148160.3) that contains a contiguous sequence which is identical to the TCUP sequence DQ324364 except for 4 bp.

Protein alignments and conserved domain analysis were used to characterize the predicted translation products of the TCUP sequence. This search revealed homology to the hAT family of transposons. The specific domains contained in TCUP include a ZnF-BED domain, and a hATC domain. The ZnF-BED domain is a zinc finger motif responsible for DNA binding and is present in many types of TEs. This domain is required for terminal inverted repeat (TIR) and subterminal repeat binding and gives the unique target specificity to the transposase (Becker and Kunze, 1997). The hATC domain is present in all hAT transposases, and is involved in dimerization of the transposase. This domain is required for proper transposase function (Essers et al., 2000). These two domains, which are required for autonomous transposition, are present in the transposases Ac and Tam3 along with other transposases in the hAT family.

The TCUP sequence is flanked by an 8-bp TSD with perfect identity, which is a characteristic of hAT transposons. The sequence also contains 13 bp imperfect TIRs that vary in sequence at the sixth base pair where there is a T in the 5’ TIR and an A at the 3’ TIR. The TIR sequences are not homologous to any previously reported transposable element indicating that this is a member of a novel, previously uncharacterized family of TEs. Analysis of the GC content of TCUP revealed a 60–80% GC content at the 5’ UTR and regions proximal of TIRs containing two CpG islands at 47–890 and 1216–1659 bp that is similar to other characterized hAT transposons. GC rich regions in TEs have been shown to be important sites of DNA methylation-dependent transcriptional regulation (Chomet et al., 1987; Kunze and Starlinger, 1989; Brutnell and Dellaporta, 1994; Brutnell et al., 1997).

Subterminal repeats are repetitive sequence motifs that are required for transposase binding that follow the TIR and are found in autonomous and non-autonomous TEs. In most transposons, subterminal repeats range in size from 5 to 12 bp, are present within inverted repeats, and can occur five to more than 50 times in some elements (Kunze and Weil, 2002). We therefore sought to identify subterminal repeats in TCUP. A CGGCAC motif was identified which is present 21 times in both forward and reverse complement orientations in the subterminal regions. The reverse of this repeat, CACGGC, is also present 18 times in both the forward and reverse complement orientations. Based on its abundance and location in the element, the CGGCAC/CACGGC sequence is the most likely candidate for a transposase binding site.

The copy number of TCUP was assessed by BAC library screening and DNA gel blot analysis using 5’, 3’, and internal segments of TCUP as probes, and by informatics analysis of the AGP version 2 genome assembly. The ZMMBB BAC HindIII B73 library (Tomkins et al., 2002) covering 14 genome equivalents was screened by hybridization using the TCUP internal probe. The results from the BAC library screen suggest that an estimated 17 copies (233 positive BACs/14 genome equivalents) that hybridize to the TCUP internal sequence are present in the B73 genome. The banding pattern of DNA gel blots digested with the restriction enzymes EcoRI, HindIII, SspI, and SwaI detected between 13 and 16 partial copies using TCUP5’, TCUP internal, or TCUP3’ probes (Figure 2). Further analysis of fragment size and band intensity on the DNA gel blots provided an estimate of three to five putative full-length elements based on the presence of an equivalent sized band across...
all probes. Evaluation of the AGP version 2 assembly using a minimum e-value cut off of $10^{-10}$ and minimum identity of 75 identified 70 (DQ324364.1) or 64 (TCUP cds) fragments in the genome that are greater than 500 bp without another fragment within 3000 bp of said fragment. Of these fragments, two putative autonomous elements on chromosomes 7 and 8, and 6 putative non-autonomous, but nearly full-length, elements on chromosomes 1, 3, 4, 9, and 10 were identified in the maize genome based on the presence of TIRs and TSD sequences. The putative autonomous elements contained uninterrupted TCUP transposase ORFs (Table 1). The number of putative autonomous elements identified by in silico analysis of the maize genome agrees with the number of sequences observed through DNA gel blot analysis (Figure 2).

**Table 1 | Summary of autonomous TCUP elements in the maize genome (AGPv2).**

| Sequence | 5' TIR | 3' TIR | TSD | Chr | 5'TIR Start | 3' TIR Stop | Length |
|----------|--------|--------|-----|-----|-------------|-------------|--------|
| DQ324364* | TATAGTTGCCAA | TTGCCCATCTATA | AAGCAAG | 7 | 115988344 | 115992684 | 4341 |
| TCUP-7a1 | TATAGTTGCCAA | TTGCCCATCTATA | CCTGGCGG | 7 | 167041903 | 167046049 | 4147 |
| TCUP-8a1 | TATAGaTTGCCAA | TTGCCCaCTATA | TCAGTGCGG | 8 | 167041903 | 167046049 | 4147 |

*TCP sequence determined by targeted sequencing and BAC information prior to whole genome assembly. Position in AGPv2 does not represent actual length due to assembly of BAC ZMMBB0310A01 which is inconsistent with our sequence.

**PHYLOGENETIC ANALYSIS REVEALS RELATED SORGHUM AND RICE TCUP HOMOLOGS THAT ARE EXPRESSED IN CULTURED CELLS AND REGENERATED PLANTS, RESPECTIVELY**

Phylogenetic analysis was performed on the putative TCUP transposase to examine its relationship to a diverse set of hAT transposases (Figure 3). Sequences included in this alignment were chosen to sample the diversity of hAT elements present in both plant and animal genomes (Xu and Dooner, 2005). An alignment of hAT transposases was used to determine the relationship of TCUP to groups of transposases within the hAT superfamily of elements. The interpretation of the grouping of the transposases was based on Xu and Dooner (2005) in which the authors surveyed a large sample of hAT transposases and categorized them into five groups (Figure 3). This analysis places TCUP within the Ac/Tam3 group of transposases in the hAT family. The TCUP transposase clusters with a group of uncharacterized sequences from rice and sorghum that are most closely related to Tam3.

EST distribution for the most closely related sequences was evaluated in the TIGR Gene Indices (ZmGI release 15.0). Interestingly, the most closely related sorghum sequence TC93360 (SbGI release 8.0) is represented only by ESTs from tissue culture and the most closely related rice (O. sativa japonica) sequences TC253370 and TC267041 (OsGI release 16.0) are represented only by ESTs from regenerared plants. This suggests that this family of transposons may have a conserved sensitivity across grasses for activation under the conditions imposed by tissue culture.

The rice (O. sativa japonica) genomic sequence at NCBI was queried to identify putative TCUP homologs. Seventeen different rice sequences with amino acid homology to the TCUP transposase were identified. The rice transposases, when compared to the maize TCUP element’s predicted hAT transposase ORF, had 53 and 54% identical and 67 and 72% conserved residues. The identified rice sequences were separated into two distinct groups based on protein and nucleotide sequences. The two groups of rice transposases were 71% identical and had 82% conserved amino acid residues relative to each other. Group I is comprised of six sequences 4450 bp in length encoding a 756 amino acid putative transposase. Group II contains 11 sequences 3916 bp in length encoding a 763 amino acid putative transposase. Analysis of the rice transposases found that several elements from each group still encode uninterrupted open reading frames containing Znf-BED and hATC domains. The majority of these sequences however appeared to be derived from immobile
elements lacking the necessary sequence motifs for transposition or non-autonomous transposons that no longer contain transposase ORFs. The terminal regions of the Group I and Group II sequences are very similar with Group I possessing 20 bp perfect TIRs and Group II having 17 bp perfect TIRs. The TIRs from these two groups of elements are also most identical and vary at two base pairs along their regions of homology (Figure 4). A 12-bp core region of the TIRs is conserved among the rice and maize TCUP elements (Figure 4). This core region has an A/T variation at the sixth base pair between the different groups of rice elements.

**FIGURE 3** | Phylogenetic analysis of hAT transposases from multiple genera. The accessions chosen for this tree were based on previous analysis performed by Xu and Dooner (2005). The different clades of hAT transposases are marked with brackets. The values in each bracket represent percentage of times the clade is present among 1000 bootstraps.

**FIGURE 4** | Comparison of maize TCUP and rice TCUP-like TIRs. Rice elements are grouped according to size. TIR sequences identical to the maize TCUP sixth position are in bold. The core 12 bp of similarity is boxed. Sequences not matching the consensus are shaded in black.
that is at the same position as the A/T variation in the imperfect TIRs of TCUP.

The TCUP transposase protein sequence was used to search the genome sequences of *S. bicolor* (sorghum), *Setaria italica* (foxtail millet), *Populus trichocarpa* (poplar) and *A. thaliana* for closely related sequences. If a protein sequence was identified with at least 40% homology to the maize TCUP transposase, the surrounding 10 kb of DNA sequence was evaluated to identify TIRs. TCUP-like sequences were identified in sorghum and millet, but not in poplar (JGI assembly release 1.0) and *A. thaliana* (TAIR9 assembly). TCUP sequences encoding putative transposase ORFs with up to 79% identity and 87% conserved amino acids was identified in the sorghum genome (Sbi1.4) assembly on chromosomes 5, 8, 9, and 10. These sequences were evaluated and contained a putative TIR sequence that was identical to the maize TCUP TIR. The foxtail millet genome assembly (JGI assembly release v1.0) also contained TCUP-like sequences encoding putative transposable ORFs with up to 79% identity and 88% conserved amino acids.

**TCUP EXPRESSION IS INCREASED BY TREATMENT WITH THE DNA METHYLATION INHIBITOR 5- AZA-2-DEOXYCYTIDINE AND MULTIPLE ISOFORMS ARE EXPRESSED**

Epigenetic derepression of transposons has been implicated as a mechanism underlying transposon activation. We conducted an experiment using the DNA methyltransferase inhibitor, 5-aza-2-deoxycytidine (azadC) to test whether this treatment can activate TCUP. Previous studies across various organisms (maize by Pan and Peterson, 1989, snap dragon by Martin et al., 1989, and human cell lines by Davis et al., 1989; Neidhart et al., 2000) provide a precedent for using azadC to activate TEs through its inhibitory action on DNA methylation. Suspension cell cultures were prepared from newly initiated Hi-II callus cells. The suspension cultures were split into two groups, a control group and a 5-aza-2-deoxycytidine (azadC) treatment group with three replicates per treatment group (Figure 5). The effectiveness of the 5-aza-2-deoxycytidine treatment in reducing DNA methylation was verified using a DNA gel blot of *HpaII* digested DNA probed with a 5S rDNA probe (Figure A1 in Appendix). DNA gel blots containing genomic DNA of azadC treated callus cultures were used to assess changes in DNA methylation of TCUP sequences caused by the azadC treatments relative to untreated callus. A reduction in CpG DNA methylation at the 5S locus was observed in the *HpaII* digested DNA from the azadC treated cultures but not in the untreated control cultures. A reduction in methylation was detected in *HpaII* digested DNA but the band is faint indicating that either a portion of cells in the callus cultures were demethylated and/or that a specific copy of TCUP was demethylated (Figure A2 in Appendix).

RT-PCR analysis of RNA isolated from suspension cultures treated with azadC, untreated suspension cultures, long-term cultured BMS callus, and whole plant seedlings was used to assess TCUP expression. The initial experiments to test for induction by azadC treatments yielded multiple transcripts in azadC treated cultures and a single transcript in BMS cultures. To further characterize these transcripts and identify if a transcript containing an intact ORF was produced, both 5’ and 3’ RACE were performed on treated suspension cultures and long-term BMS callus to identify transcription start sites and polyadenylation sites. DNA sequencing of cloned RACE PCR products identified the presence of multiple transcripts in azadC treated suspension cultures with at least three different polyadenylation sites at 3785, 3810, and 3994 bps, and an abundant BMS transcript with a polyadenylation site at 3776 bps of the TCUP transposon sequence DQ324364 was identified (Figure 1; Figure A3 in Appendix). Based on the results of the sequenced RACE PCR products, primers were designed to common sequences of the azadC and BMS RACE transcripts. RT-PCR was again performed on the suspension culture treated with azadC, untreated suspension cultures, long-term cultured BMS callus, and whole plant seedlings to confirm the observed TCUP expression pattern (Figure 6). These results indicate that some proportion of the transcripts produced in the azadC treated cell cultures are related to transcripts capable of encoding a functional transposase while the major transcript present in the long-term BMS cell culture contain an internal deletion and do appear capable of producing function TCUP transposase.
EVIDENCE OF TCUP TRANSPOSITION

_Tissue Culture Up-Regulated_ transcription was detected in long-term maize cell lines. We therefore sought to determine if TCUP also transposed in long-term cultured cells. Several independent Hi-II A × B cell lines ranging in age from 2 to 3 years following initiation and a 28-year long-term BMS cell line were analyzed for transposition using transposon display. Since cultured cells contain two homologous chromosomes and may be a mosaic of cell types, it is most likely that transposition events will appear as new bands without loss of existing bands. Transposon display using 3' specific primers detected an increase of four copies of TCUP in line H6 and an increase in copy number in BMS tissue culture (Figure 7). New insertions were not detected in callus lines I2, I3, I5, I6, I9, I10, H3, and seedlings from genotypes Hi-II-A, Hi-II-B, Hi-II-A × B, B73, BMS + B chromosomes, BMS − B chromosomes. Transposon display using the 5' specific primers did not detect any changes or mobility of TCUP. The new copies of TCUP were present in all three H6 culture biological replicates, indicating that the transposition likely occurred near the time of culture initiation. It is not possible to determine the timing of transposition in the BMS cell lines.

One of the novel bands in the H6 lines was confirmed to be novel TCUP insertions byexcising the novel bands, reamplifying the PCR product, and sequencing the cloned PCR products. The sequenced clone contained 35 bps of TCUP sequence containing the primer sequence and 11 bps of TCUP sequence that corresponded to the TIR except for a C/T transversion at the fifth position in lines H6-2, H6-3, and H6-4 and 222 bps of genomic DNA sequence. Primers were designed from the flanking genomic sequence and PCR was performed using both the 3' primary and nested TCUP specific primers. Amplification was detected in H6 lines but not in B73, Hi-A × B, BMS callus, and two independent Hi-II A × B callus lines with both primers indicating the uniqueness of this insertion (Figure 8).

DISCUSSION

TCUP IS A NOVEL MAIZE hAT TRANSPONSON

_Tissue Culture Up-Regulated_ expands the list of TEs that are active in maize and is different from previously reported transposons as indicated by its novel TIRs. Activation of transposons by tissue culture is a common occurrence in plant cell culture, but does not
plants (Peschke et al., 1987, 1991). In rice, the transposons occurred at a frequency of 3 and 1% in regenerated tissue culture than

Epigenetic changes are associated with the tissue culture process and have been implicated as a significant source of somaclonal variation. Epigenetic changes are reversible alterations in phenotype or gene expression that are stably inherited through mitosis or meiosis and not due to changes in primary DNA sequence. The mechanisms that cause the epigenetic repression or cycling of gene expression have been attributed to DNA methylation, RNA interference, and modification of histone tails. These epigenetic pathways are critical in repressing the transcription of repetitive sequences such as TEs (Chomet et al., 1987; Banks et al., 1988; Lippman et al., 2003, 2004; Zilberman et al., 2003). DNA methylation and RNA interference pathways are linked in plants by RNA dependent DNA methylation that is involved with the regulation of heterochromatin and gene silencing (Mette et al., 2000). Transcriptional repression of TEs, such as class 2 transposons, is associated with the production of 24 nt siRNAs, DNA methylation of the corresponding genomic sequence, and H3K9me2 (Lippman et al., 2004; Zilberman et al., 2007; Tanurdzic et al., 2008; Cantu et al., 2010).

Changes in the pattern of DNA methylation are thought to be a major source of epigenetic variation leading to somaclonal variation (Kaeppler and Phillips, 1993; Phillips et al., 1994; Kaeppler et al., 2000). Tissue culture induction is known to cause a general shift toward a hypomethylated genome (Kaeppler and Phillips, 1993; Koukalova et al., 2005; Law and Suttle, 2005) likely through the loss of DNA methylation of repetitive sequences within heterochromatin rather than genic sequences (Tanurdzic et al., 2008). The age of a culture is also associated with increased somaclonal variation which may be due in part to a gradual overall demethylation of low copy sequences and subsequent activation of TEs (Fukui, 1983; Hirochika et al., 1996). Increased transpositional activation of Ac is associated with tissue culture induced demethylation and increased with tissue culture age (Dennis and Brettell, 1990). The observation of TCUP transcription in long-term BMS cell cultures and azadC treated cell cultures is consistent with the role of transposons in somaclonal variation.

Both class 1 retrotransposons and class 2 transposons are known to become active from biotic and abiotic stresses. Some of the class 2 transposons known to be activated by tissue culture are Ac (Peschke et al., 1987), Spm (Peschke et al., 1991), Slide (Grappin et al., 1996), and Ping (Kikuchi et al., 2003). Retrotransposons are also activated by tissue culture stress and the majority of TEs known to be activated by tissue culture fall into this group. These activation events are associated with changes in DNA methylation. They include tobacco elements Tnt1 (Grandbastien et al., 1989) and Tio1 (Hirochika, 1993), and the rice elements TosI7 (Hirochika et al., 1996) and Karma (Komatsu et al., 2003).

The activation of TEs could be one consequence of altered DNA methylation patterns. Important interactions occur between transposases and methylated cytosine residues in transposon regulatory sequences. This is true of both Ac/Ds and En/Spm elements, where transpositional activity is dependent on the level of DNA methylation (Chomet et al., 1987; Banks et al., 1988). The transpositional activity of Ac is strongly correlated with DNA methylation present at the 5’ TIR and several different Ac alleles exist because of the different cytosine residues methylated in the subterminal repeat region of the element (Chomet et al., 1987; Brutnell and Dellaporta, 1994; Brutnell et al., 1997). Changes in DNA

EPIGENETIC CHANGES ASSOCIATED WITH TISSUE CULTURE MAY LEAD TO TCUP ACTIVATION

Epigenetic changes are associated with the tissue culture process and have been implicated as a significant source of

FIGURE 8 | Reamplification of the novel TCUP transposon site that was cloned and sequenced from the transposon display gel fragments. PCR was performed on undigested genomic DNA to confirm the novel sequence was present in H6 genomic DNA and not in other callus or plant genomes DNA. Lane 1 B73 seedling, lane 2 Hi-II A × B seedling, lane 3 Hi-II A × B callus line H3-6, lane 4 Hi-II A × B callus line H6-2, lane 5 Hi-II A × B callus line H6-3, lane 6 Hi-II A × B callus line H6-4, lane 7 Hi-II A × B callus line I9-9, lane 8 BMS callus.
methylaation, often observed as a decrease, occur as a result of the tissue culture process (Kaeppler and Phillips, 1993). Since TCUP was first discovered as a transcribed element in long-term tissue-cultured cell lines, possibly by the demethylation associated with tissue culture age, we used pharmacological treatments to reduce DNA methylation in young Hi-II A × B callus and test if induction of TCUP transcription may occur within a hypomethylated genomic environment.

AzadC and its analog 5-aza-cytidine (azaC) have been used to activate TEs in maize (Pan and Peterson, 1989), snap dragon (Martin et al., 1989), and human cell lines (Davis et al., 1989; Neidhart et al., 2000) through its inhibitory action on DNA methylation. Treatment with azaC was associated with new excision, integration, and/or demethylation events of endogenous Tans3 element in snap dragon (Martin et al., 1989) and activation of transcript of L1 elements in human fibroblasts treated with azadC (Neidhart et al., 2000). We hypothesized that azaC treatment would cause a general decrease in DNA methylation mimicking the effect of tissue culture age, which is associated with hypomethylation, and activate TCUP in young cell cultures. AzadC treatments caused transcription of a quiescent TCUP in treated Hi-II A × B recently cultured suspension cell lines and not in untreated controls. Further research is necessary to characterize the role of DNA methylation in TCUP expression. However, it is well established that DNA methylation plays an important role in regulating expression and movement of other class II TEs including Ac (Dennis and Brettell, 1990), Spm (Banks et al., 1988), and Mu (Chandler and Walbot, 1986) and class I retrotransposons (Hsiao et al., 1986; Hirochika et al., 2000). At least four different transcripts are detected by RTPCR, which could be related to transcriptional activation of both autonomous and non-autonomous TCUP elements through the genome wide demethylation caused by the azadC treatments. It has been observed that crossing an active Ac element into a background with silent Ds elements will induce transcription of the Ds elements (Kunze and Weil, 2002). Treatment with azadC may have activated a silenced TCUP element capable of trans activating other TCUP sequences creating the large number of different transcripts.

In summary, we have identified a novel hAT transposon with homology to Tans3 that exhibits differential transcription in long-term maize tissue-cultured cell lines. The element is also transcribed in newly initiated cultures in response to treatment with the DNA methylation inhibitor, 5-aza-2-deoxycytidine, when compared to untreated cultures. Mobility of TCUP was demonstrated in two independent long-term cell lines. TCUP has close relatives in rice and sorghum and EST evidence supports expression associated with tissue culture in these species. This research implicates TCUP and its related elements in rice and sorghum as a novel family of transposons that are highly sensitive to the tissue culture environment.

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APPENDIX

FIGURE A1 | DNA gel blot comparing DNA methylation at the 5S ribosomal DNA loci in untreated and 5-aza-2-deoxycytidine treated Hi-II A × B tissue-cultured cell lines. The plant and callus DNA was digested with HpaII to examine and CpG methylation respectively. An increase in number and intensity of low molecular weight bands in the HpaII digested DNA indicates a decrease in DNA methylation. Hi-II A × B genomic DNA isolated from 14-day-old seedlings (S), from independent untreated control cultures (C1–C3), from independent 5-aza-2-deoxycytidine treated cultures (A1–A3).

FIGURE A2 | DNA gel blot of untreated and 5-aza-2-deoxycytidine treated Hi-II A × B suspension cultures hybridized with the TCUP internal probe. HpaII digested genomic DNA of three independent untreated cultures (C1–C3) and three independent 5-aza-2-deoxycytidine treated cultures (A1–A3). An arrow marks the location of novel bands present in the HpaII digested DNA.
FIGURE A3 | Results of nested 3′RACE demonstrating the single BMS transcript in lane 2, and azadC transcripts labeled A–D (lanes 3–5). Lane 1 is a dH2O negative control. These bands were cloned and sequenced to identify the polyadenylation sites present in the TCUP transcripts. Sequencing of these PCR products along with 5′ RACE products were used to generate the primers used to amplify the transcripts in Figure 6.