Circadian Expression of the Maize Catalase *Cat3* Gene Is Highly Conserved Among Diverse Maize Genotypes With Structurally Different Promoters

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ABSTRACT

The *Cat3* gene of maize exhibits a transcriptionally regulated circadian rhythm. In the present study we examined the following: (1) the extent of the circadian *Cat3* expression between maize genotypes of diverse origin; (2) the functional significance of a *Tourist* transposable element located in the *Cat3* promoter of the inbred line W64A, which harbors putative regulatory elements (GATA repeat, CCAAT boxes) shown to be involved in the light induction and circadian regulation of the Arabidopsis *CAB2*, as well as other plant genes; and (3) aspects of the physiological role of CAT-3 in maize metabolism. Results confirm that the circadian *Cat3* expression is a general phenomenon in maize. Regulation of *Cat3* gene expression is not dependent on the presence of the *Tourist* element in the promoter of the gene nor on the presence of motifs similar to those found significant in the circadian expression of the Arabidopsis *CAB2* gene. Structural diversity was revealed in the *Cat3* promoters of maize genotypes of diverse origins. However, highly conserved regions with putative regulatory motifs were identified. Relevance of the conserved regions to the circadian regulation of the gene is discussed. Possible physiological roles of CAT-3 are suggested.

THE three maize catalase genes respond differentially to light in developing maize leaves. *Cat1* expression is light-independent (Redinbaugh *et al.* 1990), *Cat2* is positively regulated by light (Skadsen and Scandalios 1987; Acevedo *et al.* 1991), and *Cat3* is regulated by a circadian rhythm (Redinbaugh *et al.* 1990; Acevedo *et al.* 1991). Studies on several aspects of the *Cat3* circadian expression as well as indications of putative physiological role(s) of CAT-3 have been recently reviewed (Scandalios *et al.* 1997). Because studies with other plant genes have fortified the assumption that light and circadian responsive elements are located in the 5′ flanking regions of structural genes (Millar and Kay 1991; Anderson *et al.* 1994), we examined the *Cat3* 5′ upstream region for the presence of regulatory motifs. Several transcription factor binding sites and a transposable element were identified (Polidoros and Scandalios 1997). The transposable element located at −171 belongs to a large family of small inverted repeat elements designated "*Tourist*," first described in the *wx*-B2 mutation of maize. Members of this family of elements are highly repetitive in genomes of some and perhaps all members of the grasses, are associated with insertion mutations, and are also found in the introns and flanking sequences of wild-type genes (Bureau and Wessler 1994a).

Transposable elements can alter gene expression when inserted in or near genes. The most common effect is a null mutation when an element interrupts the coding region of a gene (McLaughlin and Walbot 1987; McCarty *et al.* 1989; Abler and Scandalios 1991). Insertion of an element in an intron may result in novel RNA-processing events, as in the maize *Adh1-S* gene (Luehrsen and Walbot 1990). The presence of an element in the promoter region can cause a spectrum of altered expression responses, as has been evident from paradigms in maize as well as in many other organisms. Low amounts of the *Bz* gene product (Schiefelbein *et al.* 1988), reduced levels of Wx enzymatic activity (Weil *et al.* 1992), and no detectable reporter activity of a PGT3 patatin promoter fusion to β-Glucuronidase in transgenic potato or tobacco plants (Koester-Toepfer *et al.* 1990) are some examples of negative regulation after incorporation of a transposable element in the 5′ flanking region of diverse genes. There are also examples where insertion of an element in the 5′ upstream region of the gene does not affect transcription. Comparison of the *Arabidopsis thaliana* meiosis-specific gene *AtDMC1* isolated from Landsberg erecta ecotype to its Columbia allele *ArLIM15*, revealed the presence of a 1874-bp transposon-like element within the promoter region of *ArLIM15*, located 219 bp upstream of the transcription initiation. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the expression levels of *AtDMC1* and *ArLIM15* are similar (Klimyuk and Jones 1997).

Rigorous control of gene expression by transposon element insertion in the promoter can be accomplished

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when the element carries regulatory sequences. This has been described in several examples, including mouse (Stavenhagen and Robins 1988), human (Ting *et al.* 1992), and plant genes (White *et al.* 1994). In plants, *copia*-like retrotransposon sequences have been shown to contain *cis*-regulatory elements which, in several cases, control the expression of the recipient genes (White *et al.* 1994). Members of the *Stowaway* family of plant inverted repeat elements are frequently located in 5′ upstream regions of several genes and appear to contain previously identified *cis*-regulatory domains (Bureau and Wessler 1994b). *Stowaway* elements share many structural features with the *Tourist* elements but have no significant sequence similarity.

Careful examination of the *Tourist* element located in the maize *Cat3* promoter (termed *Zm13*) revealed motifs that share striking similarities to a 78-bp *cis*-acting domain of the Arabidopsis *CAB2* promoter (Polidoros and Scandalios 1997). This domain is sufficient to confer red-light induction of a luciferase (*luc*) reporter gene in etiolated tobacco plants and rhythmic expression in green tissue (Anderson *et al.* 1994). Based on these similarities, we hypothesized that the *Tourist Zm13* transposable element present in the *Cat3* promoter of the maize inbred line W64A might represent a *cis*-regulatory sequence directing the light-inducible and circadian expression of the gene (Polidoros and Scandalios 1997).

Considering that maize is a highly polymorphic species, we set up an experimental scheme to test the above hypothesis and determine whether *Cat3* expression is under circadian control in other maize lines and whether the circadian expression of the *Cat3* gene depends upon the presence of the *Tourist* element in the *Cat3* promoter. Results obtained from these studies could also provide information on the physiological role of CAT-3. More specifically, we examined whether among diverse maize genotypes (1) the circadian regulation of *Cat3* is conserved, (2) the structure of *Cat3* is conserved, (3) the *Tourist* element is present in the *Cat3* promoter, (4) the presence of the *Tourist* element or presence of the motifs it harbors are necessary for the circadian regulation of *Cat3*, and (5) the circadian regulation of *Cat3* is associated with a specific physiological role.

MATERIALS AND METHODS

Plant material: In this study we used the following: the North American maize inbred lines W64A, KYS, Tx303, and Oh51A; A16, a line of Yugoslavian origin that is CAT-2 null; five inbred lines developed from tropical germplasm of diverse background, namely, NC296, NC298, NC300, NC302, and NC304, kindly provided by M. Goodman (North Carolina State University); and the line Black Mexican Sweet (BMS). Seeds were surface sterilized in 10% hypochlorite, soaked in distilled water overnight, and planted in soil mix or germinated in plastic containers on several layers of germination paper moistened with distilled water.

Zymogram analysis: Seeds were germinated and grown in plastic containers in darkness at 25° for 10 days. Samples were prepared from coleoptile tissue in glycylglycine buffer, catalase activity and protein concentration were determined, and gel electrophoresis of CAT isozymes was performed according to Chandlee and Scandalios (1984).

Northern analysis: RNA was isolated from soil-grown plants. Germination and subsequent growth was at 25° for 10 days in 12-hr dark/12-hr light (12D/12L) photoperiod. Leaf material was harvested the 10th day at circadian times (CT) 2, 6, 10, 14, 18, and 22, using a green safety light during the dark period. All samples were frozen in liquid nitrogen and stored at −80° for analysis. Total RNA from leaf material was extracted according to Thompson *et al.* (1983) and separated on denaturing 1.2 or 1.6% agarose gels. It was then transferred onto nitrocellulose or nylon membranes and hybridized with a *Cat3* gene-specific probe (Redinbaugh *et al.* 1988). Hybridization buffers and conditions were modifications of protocols recommended by the manual of the S&S Nytran Plus membrane (Schleicher and Schuell, Keene, NH). Briefly, the prehybridization solution contained 50% formamide, 6× SSPE, 5× Denhardt's, 7% sodium dodecyl sulfate (SDS), 200 ug/ml salmon sperm DNA, and 1% dextran sulfate. After 4-hr prehybridization at 42°, the prehybridization solution was substituted with hybridization solution. This was the same as the prehybridization solution without 5× Denhardt's, to which probe was added. Hybridization proceeded at 42° for 24–36 hr, and washes were performed twice for 30 min with 0.1× SSC/0.1% SDS at 42°.

DNA isolation and Southern analysis: Genomic DNA from each maize line was isolated from 10-day-old light-grown leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to instructions. For W64A and A16, genomic DNA was also isolated from 10-day-old light-grown leaves as described (Redinbaugh *et al.* 1988). Genomic DNA (10 μg) was digested with *Sst*I, electrophoresed through 1% agarose gel, and transferred onto S&S Nytran membranes using alkaline transfer according to the manufacturer. Hybridization and washes were as described for Northern analyses. A ^{32}P-labeled *Cat3* gene-specific probe (Redinbaugh *et al.* 1988) was used in order to examine the 3′ flanking region of the gene. A 185-bp *Apa*I-*Xho*I fragment of the *Cat3* promoter that includes the 143-bp *Tourist Zm13* transposable element was subcloned into pBluescript SK and used to detect the element in the different lines. The *Tourist* probe, prepared with PCR using the M13 reverse and M13 forward primers, was nonradioactively labeled using 1× Fluorescein-N^6-dATP and unlabeled nucleotide mix from the Random Primer Fluorescein Labeling Kit with Antifluorescein-AP (DuPont NEN, Wilmington, DE). The reaction started with 3 min denaturation at 95° followed by 29 cycles of 15-sec denaturation at 95°, 15-sec annealing at 55°, and 30-sec extension at 75°. The last extension step was for 5 min to ensure completion of the reaction. The *Tourist* probe was then hybridized and detected according to the Random Primer Fluorescein Labeling Kit with Antifluorescein-AP (DuPont NEN) instruction manual.

PCR amplification of the *Cat3* promoter, Southern analysis, subcloning, and sequencing: The structure of the promoter region of *Cat3* in the genotypes examined was analyzed with PCR and sequencing. The primers used in PCR were 5′-GGGCCCGACCATCAGACGAACAAC-3′ upper and 5′-GTG GCTGCTGGAACGGACGGAACT-3′ lower, amplifying a 556-bp fragment of the *Cat3* promoter in W64A (GenBank accession no. L05434), which includes the *Zm13* transposon element. The upper primer is located between nt 2172–2196 in the promoter and the lower at nt 2704–2727 at the junction

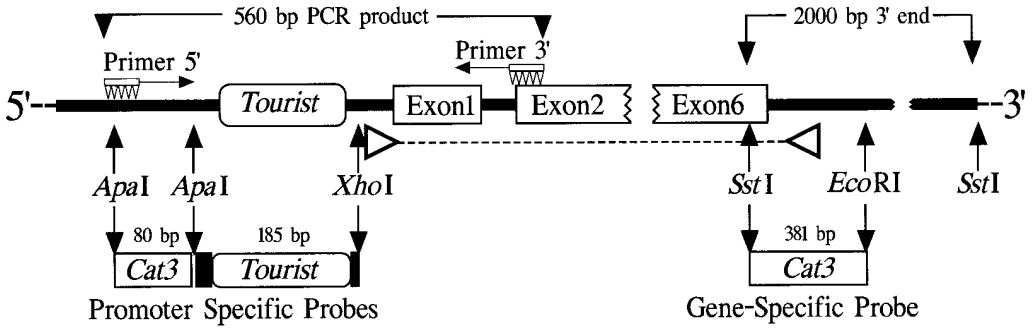


Figure 1.—Schematic of the known sequence of *Cat3* in the inbred line W64A showing features relative to this study. The combined PCR and hybridization strategy followed to detect the *Tourist* element and to determine the similarity with the *Cat3* promoter in the PCR products from the different maize genotypes is illustrated. Positions of the PCR primers and restriction enzyme borders of the promoter *Cat3*-specific, the *Tourist*-specific, and the *Cat3* gene-specific probes are shown. The illustration is not to scale, and the length (bp) of each probe or fragment is indicated.

of the first intron with the second exon of the gene. A second upper primer 5′-ATTACCGCGACGACAGGGACGATA-3′ located 54 bp 5′ of the first one was used to verify PCR results. An unrelated set of primers used to amplify the maize alternative oxidase gene (Polidoros *et al.* 1998) was also used to examine the purity of the DNA used as template in the PCR. The reactions started with 3-min denaturation at 95° followed by 35 cycles of 30 sec denaturation at 95°, 1-min annealing at 58°, and 1-min extension at 75°. The last extension step was for 5 min. The PCR products were analyzed on 1.5% agarose gels, visualized by staining with ethidium bromide, and either transferred onto nylon membranes or used for subcloning. Hybridization of the PCR products was performed with two probes: the 185-bp *Apa*I-*Xho*I fragment of the *Cat3* promoter that includes the 143-bp *Tourist Zm13* transposable element and the immediately upstream 80-bp *Apa*I fragment (Figure 1). Both probes were nonradioactively labeled and used as described for the *Tourist* probe above. To subclone the PCR products, the amplified band from each line was excised from the gel, and the DNA was isolated using the QIAquick Gel Extraction Kit (Qiagen) according to the instructions. It was then ligated to the PCR-Script SK(+) plasmid vector using the PCR-Script™ Amp SK(+) Cloning Kit (Stratagene, La Jolla, CA) according to instructions. Positive clones were identified with direct colony characterization by PCR (Gussow and Clackson 1989). PCR conditions were 3-min denaturation at 95° followed by 29 cycles of 15-sec denaturation at 95°, 15-sec annealing at 55°, and 30-sec extension at 75°, using the M13 reverse and M13 forward set of primers. The last extension step was 5 min. Plasmid DNA from positive clones was prepared for sequencing using the QIAprep Spin Miniprep Kit (Qiagen) according to instructions. Labeling and sequencing of the PCR-amplified *Cat3* promoter region of each genotype were performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Norwalk, CT) and the ABI 377 automatic DNA sequencer (Perkin Elmer).

RESULTS

Extent of *Cat3* cycling: Our results indicate that *Cat3* diurnal cycling occurs in all genotypes examined (Figure 2). The only exception is the BMS line that is null for CAT-3, because no *Cat3* message is detectable at any time point examined and no CAT-3 activity is detectable in different tissues on zymograms (S. Ruzsa and J. G. Scandalios, unpublished data). The circadian nature of the *Cat3* cycling in all genotypes was confirmed by examining *Cat3* expression in a free-running cycle. Plants grown in 12D/12L photoperiod for 10 days were subjected to constant dark for 24 hr, and *Cat3* expression was examined at the time points corresponding to the expected maximum (10 hr in constant dark) and minimum (22 hr in constant dark). Transcript levels were identical with those observed at CT 10 and CT 22 (Figure 2), respectively. The circadian rhythm of *Cat3* in the North American lines is markedly similar with high levels of expression at CT 10 and lowest expression

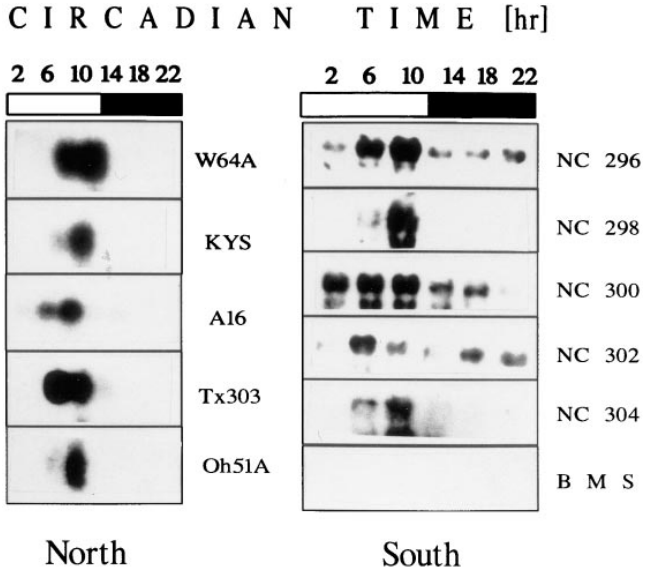


Figure 2.—Northern analysis of the circadian *Cat3* gene expression in maize genotypes of diverse origin. The left panel represents North American and the right South American genotypes. Hybridization was performed using the *Cat3* gene-specific probe. Note the similar expression pattern among the North American lines and the variant patterns among the South American lines. In all the lines *Cat3* transcripts increase during the light period and decrease during dark. No *Cat3* transcript can be detected in the CAT-3 null BMS line. Results are representative of three independent experiments.

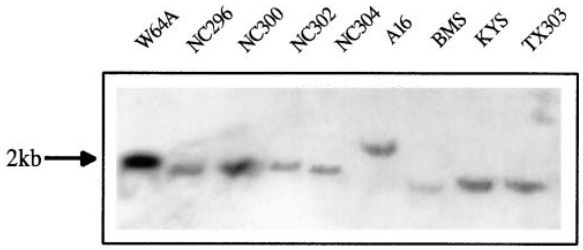


Figure 3.—Southern analysis representing restriction fragment length polymorphism (RFLP) at the 3′ end of the *Cat3* gene in diverse maize genotypes. Hybridization was performed using the *Cat3* gene-specific probe. An arrow indicates the W64A band, approximately 2 kb in length.

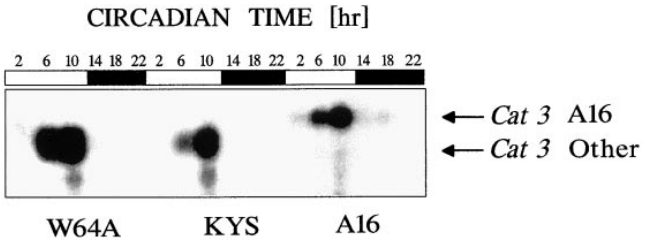


Figure 4.—Differences in transcript size for the *Cat3* mRNA among three maize lines. Northern hybridization was performed using the *Cat3* gene-specific probe. The A16 transcript is obviously longer than the transcripts of W64A and KYS, which are fairly similar.

at CT 22. A circadian expression is also present in all the NC lines, but the level of expression is quite diverse, resulting in variant patterns. Among the 5 NC lines, NC298 and NC304 display robust cycling with high levels of expression at CT 10. Lines NC296 and NC300 have a similar expression pattern with an increase in transcript very early after the onset of light, a maximum at CT 10, and lower amounts of transcript at the dark period. However, the early induction is much stronger in NC300. Line NC302 shows high expression at CT 6, slightly lower at CT 10, and constant lower expression during the dark period. In conclusion, our analysis indicates that a circadian expression of *Cat3* occurs among all the lines examined. However, differences exist with respect to the amplitude of the circadian rhythm, or the level of expression at specific times.

Modification of the 3′ end does not affect the *Cat3* circadian rhythm: We examined whether the structure of the *Cat3* gene is conserved among genetically diverse maize lines. First we examined the 3′ end of the gene using a *Cat3* gene-specific probe in Southern hybridizations of genomic DNA digested with SstI. Results indicate that extensive polymorphism exists at the 3′ end of the *Cat3* in the lines we analyzed (Figure 3). The reference line W64A gives a strong signal of the expected size at ~2 kb. In the rest of the examined lines the signal is weaker, indicating possible mismatches in the hybridization, as an equal amount of DNA was loaded on the gel (determined spectrophotometrically and after staining with ethidium bromide). One line, A16, shows a band of higher molecular weight, and the rest of the lines show variable lower MW bands than that of W64A. The higher MW of the A16 3′ end correlates to higher transcript size, whereas the lower MW of KYS does not (Figure 4). However, in all the lines, zymogram analysis indicated that the same CAT-3 isozyme (CAT-3A) is present (Figure 5). This strongly suggests that the modifications at the 3′ end are most probably located at the 3′ untranslated (for A16) and untranscribed (for the other lines) region.

These results indicate that extensive modifications in the gene structure occur at the 3′ end of the *Cat3* gene in maize genotypes of diverse origin. In connection with the conserved *Cat3* cycling in the same diverse genotypes, our results clearly indicate that modifications in the 3′ prime end of *Cat3* do not affect the cycling expression of the gene in these inbred lines.

The promoter region of *Cat3* is not conserved among different maize genotypes: To determine the presence of the *Zm13* transposon element, we used a PCR-based approach to identify the structure of the *Cat3* promoter in the genotypes examined. Results of the PCR were single band products of variable size for 10 out of the 11 lines examined, indicating that the promoter region of the gene has differences in length among these lines. In line Oh51A we failed to amplify any fragment after several attempts with different DNA isolates as templates, indicating strong sequence differences at the primer locations between W64A and Oh51A. Thus we omitted this line from further analyses. Three lines, BMS, KYS, and Tx303, had low amounts of PCR product (Figure 6). These results were identical when either of the two upper primers were used in PCR (see materials and methods). To ensure that the faint bands in BMS, KYS, and Tx303 were not because of template impurities, we used the same DNA templates in PCR with a set of test primers and under the same conditions to amplify a 500-bp fragment of the maize alternative oxidase gene (Polidoros *et al.* 1998). DNA templates from all the lines were sufficiently pure for successful amplification of the same fragment in equal quantity (Figure 6). Further analyses showed that these faint bands in BMS, KYS, and Tx303 were nonspecifically amplified

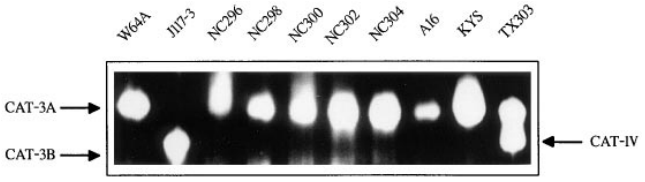


Figure 5.—Zymogram analysis indicating that the same *Cat3A* allele is present in all the lines examined in the present study. J117-3 is *Cat3B* control.

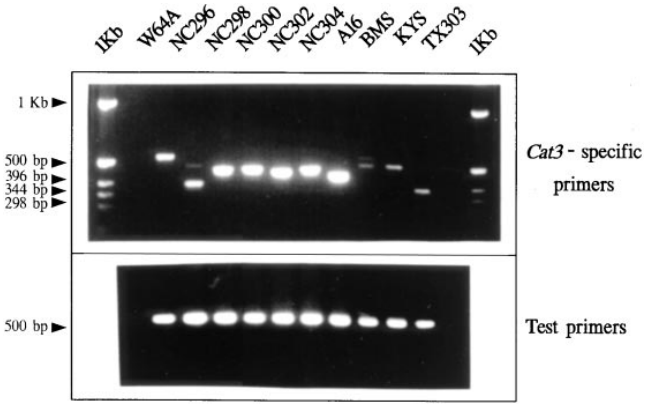


Figure 6.—Electrophoretic analysis of PCR products. Top: amplification of a promoter region of *Cat3* using DNA template from maize inbred lines of diverse origin. A single band of variable size can be detected, indicating that the promoter region of the gene differs in length among these lines. Intensity of the band is much lower in BMS, KYS, and Tx303, indicating that false product amplification likely occurred in these lines. Bottom: control PCR using a set of test primers that amplify part of the maize alternative oxidase gene and the same template DNA as in the top panel. A single band of similar size and intensity can be observed, indicating that the DNA template used in both PCRs is of sufficient purity to allow specific amplification.

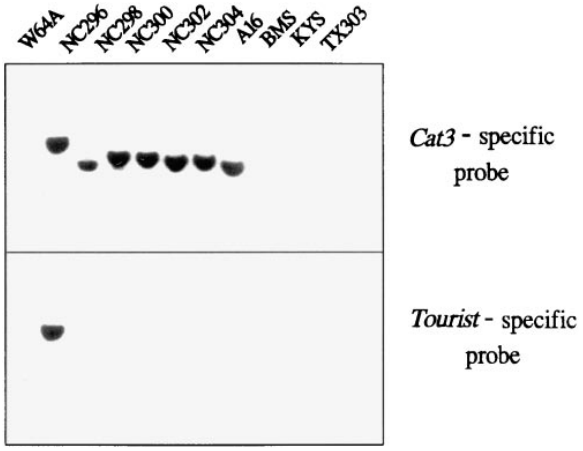


Figure 7.—Detection of similarity with the 5′ *Cat3* and the *Tourist* sequences in the PCR products from different lines. Top: hybridization of PCR bands from the different genotypes with the 80-bp *Cat3*-specific probe depicted in Figure 1. All NC lines and A16 show a strong hybridization signal. Bottom: hybridization of PCR bands from the different lines with the 185-bp *Tourist*-specific probe depicted in Figure 1. Only the W64A band hybridizes with this probe.

bands bearing no similarity to the *Cat3* promoter (see below).

The length of the PCR products in the rest of the lines was in all cases smaller than that of W64A. The difference in length is in the range of 80–100 bp except for NC296 and Tx303, where a difference of ~150 bp was detected. Because the size of the *Zm13* element is 145 bp, further analyses were required to determine the presence of the transposon element in the promoter of the *Cat3* gene in these genotypes. For this purpose we used a hybridization strategy depicted in Figure 1. Hybridization of the PCR products was performed with two probes. The 185-bp *Apa*I-*Xho*I fragment of the *Cat3* promoter that includes the 143-bp *Tourist Zm13* transposable element was used to determine the presence of the element in the different lines. The adjacent upstream 80-bp *Apa*I fragment was used to determine similarity with the *Cat3* promoter sequence of W64A. Our results indicate that, for all NC lines and A16, the amplified region is similar to the W64A *Cat3* promoter because strong hybridization signals were obtained with the 80-bp *Apa*I fragment (Figure 7, top). However, there was no detectable signal for the lines with weak PCR bands (BMS, KYS, and Tx303), indicating that possible modifications at the primer annealing sequences might cause mispriming and/or amplification of a false product. Hybridization of the same blot with the *Tourist* 185-bp *Apa*I-*Xho*I fragment of the *Cat3* promoter resulted in a strong signal only for W64A (Figure 7, bottom). This indicates that the element is missing in the *Cat3* promoter of the five NC lines and A16, whose PCR-amplified band hybridized with the *Cat3* promoter-specific 80-bp *Apa*I probe. However, multiple copies of *Tourist*-like sequences exist in the genomes of all the lines, as is evident from the multiple bands seen after hybridization of genomic DNA isolated from these lines with the *Tourist* probe (Figure 8).

Sequencing and multiple alignment of the PCR-amplified *Cat3* genomic fragments: The PCR products of the different genotypes were cloned and sequenced to determine the exact sequence of the *Cat3* promoter in these lines. Sequence analysis (Figure 9) confirmed the

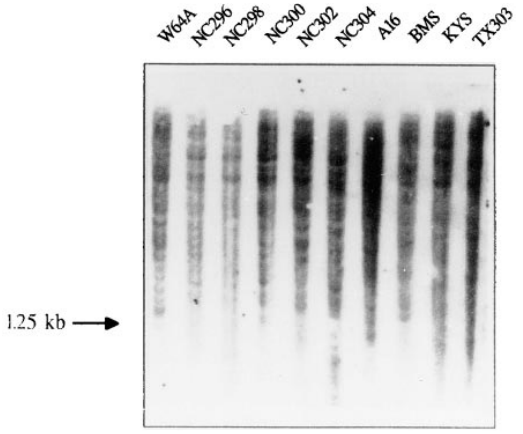
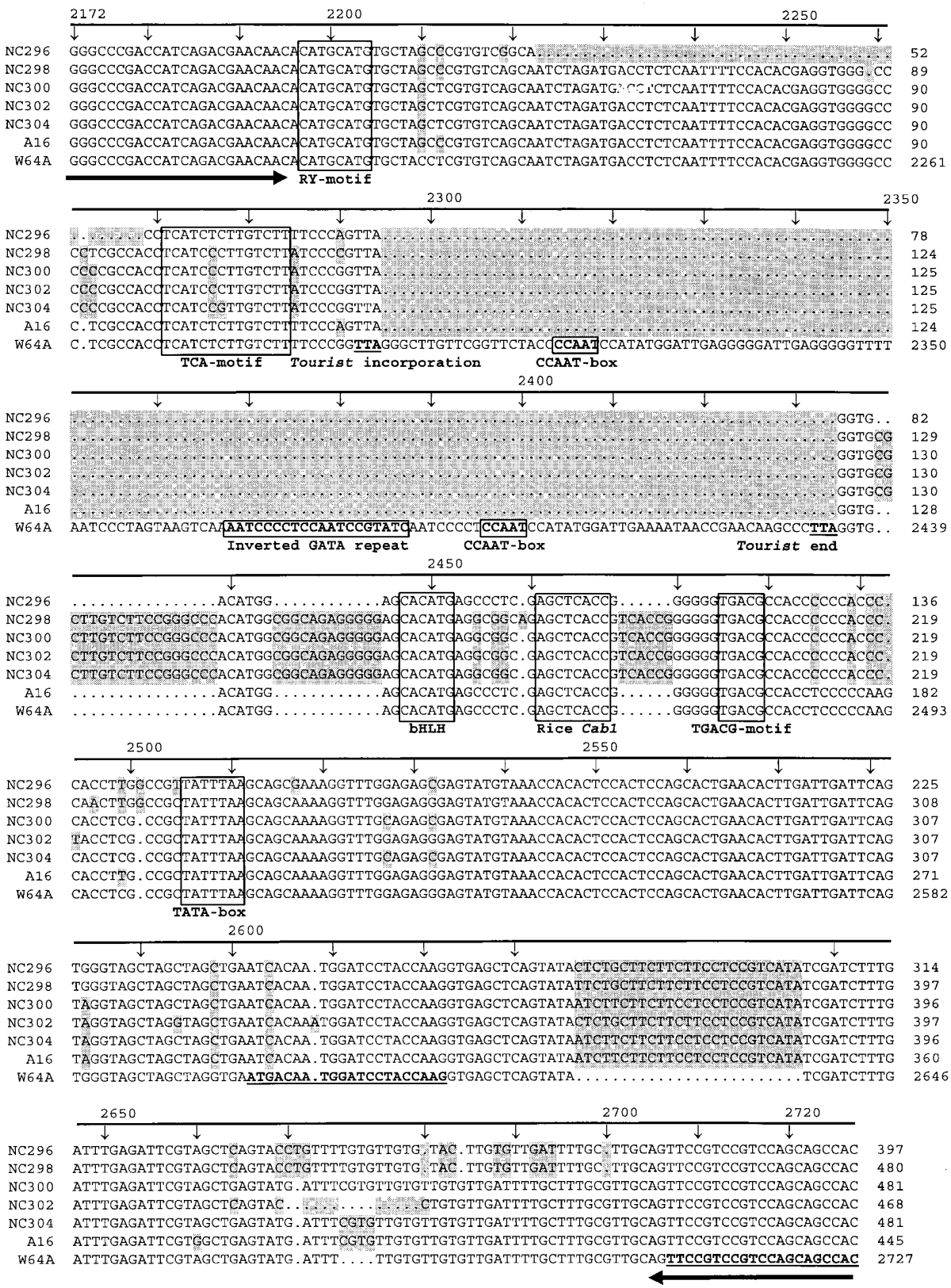


Figure 8.—Southern analysis indicating the presence of multiple copies of *Tourist*-like sequences in all the maize lines of diverse origin examined. Genomic DNA (10 μg) was digested with *Sst*I, electrophoresed through 1% agarose gel, and hybridized with the *Tourist*-specific probe. The position of the expected 1.25-kb hybridization band in W64A is indicated by an arrow.


2172
2200
2250
NC296 GGGCCCGACCATCAGACGAACAACACATGCATGTGCTAGCCCGTGTCGGCA 52
NC298 GGGCCCGACCATCAGACGAACAACACATGCATGTGCTAGCCCGTGTCAGCAATCTAGATGACCTCTCAATTTTCCACACGAGGTGGG.CC 89
NC300 GGGCCCGACCATCAGACGAACAACACATGCATGTGCTAGCTCGTGTCAGCAATCTAGATG.CCTCTCAATTTTCCACACGAGGTGGGGCC 90
NC302 GGGCCCGACCATCAGACGAACAACACATGCATGTGCTAGCTCGTGTCAGCAATCTAGATGACCTCTCAATTTTCCACACGAGGTGGGGCC 90
NC304 GGGCCCGACCATCAGACGAACAACACATGCATGTGCTAGCTCGTGTCAGCAATCTAGATGACCTCTCAATTTTCCACACGAGGTGGGGCC 90
A16 GGGCCCGACCATCAGACGAACAACACATGCATGTGCTAGCCCGTGTCAGCAATCTAGATGACCTCTCAATTTTCCACACGAGGTGGGGCC 90
W64A GGGCCCGACCATCAGACGAACAACACATGCATGTGCTACCTCGTGTCAGCAATCTAGATGACCTCTCAATTTTCCACACGAGGTGGGGCC 2261
RY-motif
2300
2350
NC296 .....CCTCATCTCTTGTCTTTTCCCAGTTA 78
NC298 CCTCGCCACCTCATCCCTTGTCTTATCCCCGTTA 124
NC300 CCCCGCCACCTCATCCCTTGTCTTATCCCGGTTA 125
NC302 CCCCGCCACCTCATCCCTTGTCTTATCCCGGTTA 125
NC304 CCCCGCCACCTCATCCGTTGTCTTATCCCGGTTA 125
A16 C.TCGCCACCTCATCTCTTGTCTTTTCCCAGTTA 124
W64A C.TCGCCACCTCATCTCTTGTCTTTTCCCGGTTAGGGCTTGTTCGGTTCTACCCCAATCCATATGGATTGAGGGGGATTGAGGGGGTTTT 2350
TCA-motif Tourist incorporation CCAAT-box
2400
NC296 GGTG.. 82
NC298 GGTGCG 129
NC300 GGTGCG 130
NC302 GGTGCG 130
NC304 GGTGCG 130
A16 GGTG.. 128
W64A AATCCCTAGTAAGTCAAAATCCCCTCCAATCCGTATCAATCCCCTCCAATCCATATGGATTGAAAATAACCGAACAAGCCCTTAGGTG.. 2439
Inverted GATA repeat CCAAT-box Tourist end
2450
NC296 .....ACATGG.....AGCACATGAGCCCTC.GAGCTCACCG.....GGGGGTGACGCCACCCCCCACCC. 136
NC298 GTTGTCTTCCGGGCCCACATGGCGGCAGAGGGGGAGCACATGAGGCGGCAGAGCTCACCGTCACCGGGGGGTGACGCCACCCCCCACCC. 219
NC300 CTTGTCTTCCGGGCCCACATGGCGGCAGAGGGGGAGCACATGAGGCGGC.GAGCTCACCGTCACCGGGGGGTGACGCCACCCCCCACCC. 219
NC302 CTTGTCTTCCGGGCCCACATGGCGGCAGAGGGGGAGCACATGAGGCGGC.GAGCTCACCGTCACCGGGGGGTGACGCCACCCCCCACCC. 219
NC304 CTTGTCTTCCGGGCCCACATGGCGGCAGAGGGGGAGCACATGAGGCGGC.GAGCTCACCGTCACCGGGGGGTGACGCCACCCCCCACCC. 219
A16 .....ACATGG.....AGCACATGAGCCCTC.GAGCTCACCG.....GGGGGTGACGCCACCTCCCCCAAG 182
W64A .....ACATGG.....AGCACATGAGCCCTC.GAGCTCACCG.....GGGGGTGACGCCACCTCCCCCAAG 2493
bHLH Rice Cab1 TGACG-motif
2500
2550
NC296 CACCTTGGCCGTTATTTAAGCAGCGAAAGGTTTGGAGAGCGAGTATGTAAACCACACTCCACTCCAGCACTGAACACTTGATTGATTCAG 225
NC298 CAACTTGGCCGCTATTTAAGCAGCAAAAGGTTTGGAGAGGGAGTATGTAAACCACACTCCACTCCAGCACTGAACACTTGATTGATTCAG 308
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NC302 TACCTCG.CCGCTATTTAAGCAGCAAAAGGTTTGGAGAGGGAGTATGTAAACCACACTCCACTCCAGCACTGAACACTTGATTGATTCAG 307
NC304 CACCTCG.CCGCTATTTAAGCAGCAAAAGGTTTGCAGAGCGAGTATGTAAACCACACTCCACTCCAGCACTGAACACTTGATTGATTCAG 307
A16 CACCTTG.CCGCTATTTAAGCAGCAAAAGGTTTGGAGAGGGAGTATGTAAACCACACTCCACTCCAGCACTGAACACTTGATTGATTCAG 271
W64A CACCTCG.CCGCTATTTAAGCAGCAAAAGGTTTGGAGAGGGAGTATGTAAACCACACTCCACTCCAGCACTGAACACTTGATTGATTCAG 2582
TATA-box
2600
NC296 TGGGTAGCTAGCTAGCTGAATCACAA.TGGATCCTACCAAGGTGAGCTCAGTATACTCTGCTTCTTCTTCCTCCGTCATATCGATCTTTG 314
NC298 TGGGTAGCTAGCTAGCTGAATCACAA.TGGATCCTACCAAGGTGAGCTCAGTATATTCTGCTTCTTCTTCCTCCGTCATATCGATCTTTG 397
NC300 TAGGTAGCTAGCTAGCTGAATCACAA.TGGATCCTACCAAGGTGAGCTCAGTATAATCTTCTTCTTCCTCCTCCGTCATATCGATCTTTG 396
NC302 TAGGTAGCTAGGTAGCTGAATCACAAATGGATCCTACCAAGGTGAGCTCAGTATACTCTGCTTCTTCTTCCTCCGTCATATCGATCTTTG 397
NC304 TAGGTAGCTAGCTAGCTGAATCACAA.TGGATCCTACCAAGGTGAGCTCAGTATAATCTTCTTCTTCCTCCTCCGTCATATCGATCTTTG 396
A16 TAGGTAGCTAGCTAGCTGAATCACAA.TGGATCCTACCAAGGTGAGCTCAGTATAATCTTCTTCTTCCTCCTCCGTCATATCGATCTTTG 360
W64A TGGGTAGCTAGCTAGGTGAATGACAA.TGGATCCTACCAAGGTGAGCTCAGTATA.....TCGATCTTTG 2646
2650
2700
2720
NC296 ATTTGAGATTCGTAGCTCAGTACCTGTTTTGTGTTGTG.TAC.TTGTGTTGATTTTGC.TTGCAGTTCCGTCCGTCCAGCAGCCAC 397
NC298 ATTTGAGATTCGTAGCTCAGTACCTGTTTTGTGTTGTG.TAC.TTGTGTTGATTTTGC.TTGCAGTTCCGTCCGTCCAGCAGCCAC 480
NC300 ATTTGAGATTCGTAGCTGAGTATG.ATTTCGTGTTGTGTTGTGTTGATTTTGCTTTGCGTTGCAGTTCCGTCCGTCCAGCAGCCAC 481
NC302 ATTTGAGATTCGTAGCTCAGTAC.....CTGTGTTGATTTTGCTTTGCGTTGCAGTTCCGTCCGTCCAGCAGCCAC 468
NC304 ATTTGAGATTCGTAGCTGAGTATG.ATTTCGTGTTGTGTTGTGTTGATTTTGCTTTGCGTTGCAGTTCCGTCCGTCCAGCAGCCAC 481
A16 ATTTGAGATTCGTGGCTGAGTATG.ATTTCGTGTTGTGTTGTGTTGATTTTGCTTTGCGTTGCAGTTCCGTCCGTCCAGCAGCCAC 445
W64A ATTTGAGATTCGTAGCTGAGTATG.ATTT....TTGTGTTGTGTTGATTTTGCTTTGCGTTGCAGTTCCGTCCGTCCAGCAGCCAC 2727

conclusions drawn from the PCR hybridization experiments. The complete *Tourist* sequence is present only in the W64A maize line. The PCR bands amplified from BMS, KYS, and Tx303 do not resemble the W64A *Cat3* promoter sequence and are not presented below. The published *Cat3* sequence from W64A (Abler and Scandalios 1993) and the amplified sequence from W64A in this report are identical. Multiple alignment of the amplified sequences of the five NC lines, A16, and W64A was performed using the MegAlign program, which is part of the Lasergene Biocomputing Software Package (DNAStar, Madison, WI) and the PILEUP program, part of the GCG Wisconsin Package (GCG, Madison, WI). The resulting alignments using either program showed that short regions with similarities between the seven lines existed within the *Tourist* element and close to its 5′ end (alignments not shown). However, most of the *Tourist* sequence was present only in W64A. The alignment downstream of the *Tourist* element (located between nt 2291 and 2434) revealed several gaps, and the seven sequences regained high similarity at position 2465 and thereafter. One possible explanation for these results could be that a *Tourist*-like element was present in ancestors of these lines, but sometimes it transposed and left behind or carried along a few nucleotides after its excision. Such phenomena are not unusual in transposition of mobile elements. However, in multiple alignments of the sequences in different combinations (each time omitting one or more lines) we observed that the NC lines and A16 were aligned better without the W64A sequence, or with W64A after deleting the *Tourist* element. This alignment indicated that the sequences of NC298, NC300, NC302, and NC304 were almost identical in a 85-bp long region immediately downstream of the *Tourist* incorporation trinucleotide TTA at position 2291. The sequences of W64A, A16, and NC296 were also identical and differed from the other NC lines as they were interrupted by three short gaps 18 bp, 12 bp, and 6 bp long. The rest of the alignments upstream and downstream of the 85-bp region were identical with the alignments obtained before the *Tourist* element was deleted from W64A and were the same using either computer program.

Alignment of the PCR-amplified *Cat3* segments was different when the *Tourist* element was retained or excised from the W64A sequence. In order to give the same alignment in the presence and the absence of the element, the computer programs should have been forced to introduce a very long gap in the other sequences, where the element is incorporated in W64A. This is an unfavorable option even after adjusting the gap penalty parameters of the programs. Thus, we simply used the alignment of the seven lines in which the sequence of the *Tourist* element was deleted, then reinserted the element in its position in the W64A sequence, and aligned with gaps in the other lines (Figure 9). The *Cat3* promoter sequences in two lines, NC300 and NC304, are exactly the same, indicating that the *Cat3* gene in these lines derived from a common ancestor. Line NC296 is the only one that is missing about 45 bp of the 116-bp region immediately upstream of the *Tourist* element location. It is also the only South American line having exactly the same gaps as A16 and W64A downstream from the *Tourist* element. Similarity between A16 and W64A is high.

DISCUSSION

Implications of the presence of a transposable element in the *Cat3* promoter: In a previous study, we reported the presence of putative circadian- and light-regulatory motifs (GATA repeat, CCAAT boxes) within the region of a *Tourist* transposable element in the *Cat3* promoter (Polidoros and Scandalios 1997). Similar motifs have been shown to be involved in the light and circadian regulation of the Arabidopsis *CAB2* gene (Anderson *et al.* 1994). This raised the possibility that circadian regulation of *Cat3* may be dependent on the presence of the transposable element (or the motifs it harbors) in the promoter of the gene. Such an assumption could have significant implications on the necessity for circadian *Cat3* expression and the physiological role of the CAT-3 isozyme in cellular metabolism. For example, if the circadian *Cat3* expression is dependent upon the presence of the *Tourist* element, then it could be a random feature due to the incorporation of the element rather than to a specific biological necessity. Based on this assumption, maize genotypes might exist that do not harbor the *Tourist* element in their *Cat3* promoter and show no circadian *Cat3* expression. Alternatively, if the circadian *Cat3* expression is because of a specific biological necessity and is independent of the presence

Figure 9.—Multiple alignment of part of the *Cat3* gene sequences resulting from PCR amplification using DNA from the maize inbred lines NC296, NC298, NC300, NC302, NC304, A16, and W64A. The genotype is shown at the left of each sequence and the nucleotide count at the right. The first nucleotide in W64A is number 2172, following the numbering of the sequence in GenBank (accession number L05934). In the rest of the lines, nucleotide count starts with number one. The length of each sequence is shown at right except for W64A, which is 655 bp. Nucleotides or sequences in shaded boxes represent disagreement with the W64A sequence. The ruler on top of the sequences shows the nucleotide count in W64A in increments of 10. Conserved motifs are shown in open boxes, and their names are in bold below the boxes. The PCR primers are shown as bold arrows below the sequence of each primer. The two exons of the *Cat3* gene in W64A are in bold and underlined. The *Tourist* incorporation target site TTA and its end site (duplicated) TTA are in bold, underlined, and indicated underneath. Within the *Tourist* sequence the CCAAT boxes and the inverted GATA repeat (which includes one additional CCAAT box) are in bold, in open boxes, and indicated below.

of the *Tourist* element, then genotypes might exist that maintain circadian *Cat3* expression in the absence of the *Tourist* element in the *Cat3* promoter. It is obvious that proving either of the above possibilities would have significant implications on the evolutionary origin of the *Cat3* circadian expression, as well as for the biological significance of this regulation.

Examination for a circadian expression of *Cat3* in maize genotypes of diverse origin in relation to the presence/absence of the *Tourist* element in the *Cat3* promoter: To study the functional significance of the *Tourist* element in the *Cat3* promoter we sought to take advantage of the highly polymorphic maize genome and the availability of diverse maize genotypes. Among these we could likely identify genotypes that harbor no *Tourist* element in the *Cat3* promoter and/or genotypes in which the expression of *Cat3* is not circadian. In fact, after examining three additional maize inbreds, Acevedo *et al.* (1991) concluded that neither *Cat3* allelic variation nor genetic background alters the circadian pattern of *Cat3* mRNA accumulation in the leaves of these lines. However, at the time this study was conducted, it was not known that a transposable element, harboring putative circadian- and light-regulatory motifs, is present in the *Cat3* promoter of W64A. Accordingly, it was suggested that the circadian regulation of *Cat3* transcription in W64A (Redinbaugh *et al.* 1990) is likely a general phenomenon in maize. This situation was questioned when the *Tourist* element was discovered in the *Cat3* promoter and the putative circadian- and light-regulatory motifs were identified on the element (Polidoros and Scandalios 1997).

To further investigate the general nature of the *Cat3* circadian expression we expanded the analysis in 10 additional maize inbreds of diverse origin (see materials and methods). Northern hybridization analysis confirmed that the circadian *Cat3* expression is conserved and has the same pattern among all these diverse genotypes (Figure 2). Differences were present regarding the amplitude of the rhythm and the expression level at different time points, especially between the South American genotypes, but this could be the result of modifications at the 3′ end of the gene, where the *Cat3* gene-specific probe used in this study hybridized. However, the same general expression pattern with the transcript increasing during the light period, and decreasing during the dark, was obvious in all the lines. Significantly, we also demonstrated that modifications of the 3′ end of the gene in these genotypes (Figure 3) did not affect the circadian expression of the gene. These modifications did not result in CAT-3 allelic variation because the same *Cat-3A* allele was present in all the lines (Figure 5). However, the transcript size was affected in A16 (Figure 4). Taken together, the above results suggest that the circadian regulation of *Cat3* transcription is a general phenomenon in maize, validating our previous assumptions (Acevedo *et al.* 1991). After ensuring the *Cat3* circadian expression in these genotypes, we asked whether it correlates with the presence of the *Tourist* element in the promoter of the gene. Combined results from PCR amplification of a *Cat3* promoter region that includes the *Tourist* element in W64A and hybridization of the PCR products with *Cat3*-specific and *Tourist*-specific probes (Figures 6 and 7) proved that none of the South American lines nor A16 harbored the *Tourist* element in the *Cat3* promoter. Thus, we could safely conclude that the presence of the *Tourist* element was not necessary for regulating the *Cat3* circadian expression. We also observed PCR-product length variability, which could not be explained solely by the absence of the *Tourist* element in all the lines. Along with our inability to amplify a promoter region in some of the lines, this was strong evidence that the *Cat3* promoter had extensive differences in the genotypes examined. The conclusion from this analysis is that incorporation of the *Tourist* element in the *Cat3* promoter of W64A does not appear to affect the circadian regulation of the gene. However, we cannot rule out the possibility that motifs similar to those present in the *Tourist* element (GATA repeat, CCAAT boxes) in W64A are also present and important for the circadian regulation of *Cat3* gene expression, but located elsewhere in the promoter of the *Cat3* in the rest of the lines. To examine this possibility, as well as to determine the exact *Cat3* promoter structure, we sequenced the PCR-amplified *Cat3* promoter region in the South American lines, A16, and W64A.

Sequence analysis and *Cat3* promoter structure: The sequences of the *Cat3* promoter from each of the seven genotypes, derived by sequencing of the PCR fragments, were aligned as described in results and analyzed for the presence of regulatory motifs. The alignment (Figure 9) confirmed the following: The sequence of the *Cat3* promoter has highly conserved and highly variable regions among the seven diverse lines examined. Relative to the differences among these lines is the absence of a *Tourist* transposable element in lines other than W64A. Significantly, no motifs similar to those present in the *Tourist* element, and presumed to play a role in the circadian regulation of *Cat3* (GATA repeat, CCAAT boxes), were present in the promoter of any other line. This suggests that other regulatory elements are responsible for the circadian regulation of the maize *Cat3* gene and indicates that differences might exist between monocot (maize) and dicot (Arabidopsis) circadian regulatory components.

The first translatable ATG triplet in W64A (Redinbaugh *et al.* 1988) is not conserved in the rest of the lines. Instead, the ATG of the third amino acid in the first *Cat3* exon of W64A seems to be the first translatable ATG in all the other lines, as it is in 14 of the 15 other plant catalase transcripts reported (Guan and Scandalios 1996). There is a 24-bp sequence with CTT repeats and a 4-bp stretch absent in W64A in the first

intron and present in all other lines. Some single base mismatches can be observed sporadically and at very low frequency in pairwise alignments between W64A and the rest of the lines. The region upstream of the element is identical between W64A and A16. Most divergent is NC296 that is missing ~45 bp of the 116 bp of the region. The rest of the NC lines have a few random single base mismatches.

Among the similarities between the lines are several highly conserved regions and putative regulatory motifs in the *Cat3* promoter sequence (Figure 9). The region between the TATA-box and the first exon is almost identical in all the lines. Upstream of the TATA-box in a short 40-bp region, the following are conserved: a TGACG motif that is a component of the promoters of many plant, animal, and viral genes (Bouchez *et al.* 1989; Inoue *et al.* 1991), serving as a binding site for basic-leucine-zipper (bZIP) transcription factors and mediating responses to auxin and salicylic acid (SA) (Miao and Lam 1995; Rouster *et al.* 1997); the light-responsive element AGCTCACC of the rice *Cab1* gene (Luan and Bogorad 1992); and a basic helix-loop-helix (bHLH) transcription factor binding site (Pabo and Sauer 1992). This is the only conserved region, between the genotypes examined, that harbors a light-responsive element and may be important in the circadian regulation of *Cat3*. Upstream of the *Tourist* location there is one motif resembling the TCA element (TCATCTT CTT), which is the binding site of the salicylic acid-inducible TCA-1 regulatory protein (Goldsbrough *et al.* 1993). In moderate concentrations, SA can induce catalase genes, and a developmental stage-specific *Cat3* induction has been observed during germination of immature developing maize embryos treated with SA (Guan and Scandalios 1995). The TCA motif at nt 2270 has a conserved TCATC part and a variable (C/T) (C/G)TTGTCTT part and may mediate the SA responses in *Cat3* transcription. Further upstream (nt 2198) there is a conserved RY motif (CATGCATG) (Baumlein *et al.* 1992; Lelievre *et al.* 1992). This motif merits special attention. The RY motif CATGCATG is present in the 5′ upstream region of a large number of plant genes mostly expressed during seed development (Baumlein *et al.* 1992). It is also present as the *Sph*I element in the SV40 enhancer (Zenke *et al.* 1986) and at the promoter of the rice *CatA* catalase gene, a rice homologue of the maize *Cat3* (Higo and Higo 1996). Destruction of this motif by short internal deletions nearly abolishes the function of a C1 maize gene (Hattori *et al.* 1992) and the legumin B4 gene of *Vicia faba* (Baumlein *et al.* 1992). Strong positive effects have also been reported for glycinin (Lelievre *et al.* 1992) and β-conglycinin (Chamberland *et al.* 1992) expression in soybean. In contrast, in *Vicia faba* the RY motif of the *usp* gene promoter exerts a silencing effect because its destruction increases transcription (Fiedler *et al.* 1993). It has been hypothesized that positive or negative effects of the RY motif in transcription are controlled by the presence of enhancers or silencer-like elements upstream of the motif (Baumlein *et al.* 1992; Fiedler *et al.* 1993). In addition, the hypothesis is supported by a number of reported unsuccessful attempts to demonstrate binding of nuclear proteins to the RY motif (Riggs *et al.* 1989; Meakin and Gatehouse 1991). A role of the RY motif in repression of *Cat3* transcription could place it as a component of the circadian regulatory apparatus of the gene as the circadian *Cat3* expression has been proposed to be the result of a cyclic repression superimposed on a quasi-constitutive mode of expression in maize leaves (Acevedo *et al.* 1991).

Downstream of the RY motif there is a sequence between nt 2241 and 2276 that is present in the rice *CatA* gene promoter (*CatA* is a homologue to the maize *Cat3*) and proposed to be specific to the promoter of the gene encoding the CAT-3 type catalase of monocots (Higo and Higo 1996). This region of unknown function is almost totally missing from NC296 and is highly conserved in the rest of the lines.

Unraveling the structural variability of the *Cat3* promoter might have important implications. Even if the circadian expression of the gene is conserved among these genotypes with structurally different promoters, other aspects of the regulation of the gene remain unknown. The W64A *Cat3* gene has been shown to respond to diverse stress conditions and challenges like the fungal toxin cercosporin (Williamson and Scandalios 1992), salycilic acid (Guan and Scandalios 1995), diverse antioxidants, H_2O_2, and mitochondrial respiration pathway-specific inhibitors (A. N. Polidoros and J. G. Scandalios, unpublished results). It is currently unknown whether *Cat3* responds similarly in other genotypes.

Aspects of the physiological role of CAT-3: The existence of CAT-3 null maize lines with no major discernible physiological defects under normal growth conditions (Scandalios *et al.* 1997) suggests that CAT-3 is a dispensable catalase isozyme. However, this does not imply that it has no specific physiological role in cellular metabolism, or that it may not be critical under adverse environments. Results presented in this study make apparent that circadian *Cat3* expression is the rule in maize. The pattern of the *Cat3* circadian rhythm in all the lines examined, with maximal mRNA accumulation late in the light period and minimal mRNA accumulation late in the dark, suggests that there might be a high demand for CAT-3 during the early dark period of the plant's metabolism. This distinguishes maize *Cat3* from the *Arabidopsis thaliana CAT2* (Zhong *et al.* 1994) and *Nicotiana plumbaginifolia Cat1* (Willekens *et al.* 1994), in which the phase of accumulation of catalase transcripts early in the light period coincides with that of photosynthetic gene transcripts and is in good agreement with a potent physiological role of catalase in decomposing H_2O_2 generated during the photorespi-

ratory oxidation of glycolate in the peroxisome. The presence of a catalase in a C3 dicot plant (*A. thaliana CAT3*) exhibiting a circadian expression phase similar to *Cat3* of maize (a C4 monocot plant) (Zhong and McClung 1996) might imply a common metabolic necessity in C3 and C4 as well as in monocot and dicot plants for catalase during the late light and early dark period. However, Arabidopsis *CAT3* has a consensus peroxisomal targeting signal and is expected to be localized in peroxisomes (Frugoli *et al.* 1996), whereas maize CAT-3 is mitochondrial (Scandalios *et al.* 1980). Thus, the nature of such a common metabolic requirement remains speculative.

Basically, two hypotheses have been proposed to explain the physiological role of CAT-3. One considers the high peroxidatic activity of CAT-3 and the overlapping histological patterns of lignification and catalase distribution in maize stem cells (Acevedo and Scandalios 1991). This suggests that CAT-3 may play a role in the lignification process providing the required peroxidatic activity. Several enzymes of the general phenylpropanoid pathway, which is part of the lignin biosynthetic pathway, are light-inducible, both at the mRNA (Ohl *et al.* 1990; Feinbaum *et al.* 1991; Kubasek *et al.* 1992; Ohl *et al.* 1990) and the enzyme activity levels (Wilkinson and Butt 1992). The activity of these enzymes reportedly showed a 1–5-hr lag before increasing after illumination in etiolated pea epicotyls, reaching a maximum over a 12-hr period when lignification was more rapid and declining thereafter. This time frame is in good agreement with the increase of *Cat3* expression 4–6 hr after the onset of light reaching a maximum after 10 hr and declining thereafter.

The second hypothesis suggests that there might be increased demand for catalase late in the light period and early in the dark period, because of an excessive H_2O_2 buildup during this period of the plant's metabolism. The excessive H_2O_2 can be attributed to decreased ascorbate peroxidase activity (Abler and Scandalios 1994), or to a diurnal fluctuation in the production of reactive oxygen species in the mitochondria of mesophyll cells (Polidoros and Scandalios 1997). The circadian regulation of *Cat3* expression fits a model of excessive H_2O_2 formation during the late light-early dark period, but this has to be examined. Further investigations are underway to examine the above hypotheses on the physiological role of maize CAT-3, the necessity of which has been implied by the results presented in this study.

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