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Isolation, characterization and expression of the maize *Cat2* catalase gene

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Abstract

The maize *Cat2* gene was isolated by direct cloning and PCR. The clones were mapped and sequenced. The start site of transcription was determined by primer extension. Computer analysis of the 1.6 kb *Cat2* promoter sequence has revealed an obvious TATA box, two GC boxes, a putative GA response element, and several ACGT core sequences known to have diverse regulatory functions in plants. Several other protein binding motifs were also identified within 800 bp upstream from the transcriptional start site. Five introns were identified in the *Cat2* coding region. All five *Cat2* introns are located in exactly the same position as five of the six introns in *Cat1*. Two of the *Cat2* introns are located in the same position as the two *Cat3* introns. The identical positioning of these introns suggests an evolutionary link between all three maize catalase genes. The response of the *Cat2* gene to plant growth regulators was examined. Results clearly showed that the response of *Cat2* to several environmental factors are developmental stage-dependent. Thus, complex regulatory mechanisms appear to be involved in the regulation of *Cat2* expression in maize.

Introduction

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6; CAT) is a tetrameric, heme-containing enzyme found in all aerobic organisms. It provides protection against reactive oxygen toxicity by dismutating hydrogen peroxide to water and oxygen [1]. In maize (*Zea mays* L.), three unlinked structural genes, *Cat1*, *Cat2*, and *Cat3* encode three biochemically distinct isozymes, CAT-1, CAT-2, and CAT-3 [2, 3]. Each of the *Cat* genes exhibits temporal and spatial specificity in its expression [4, 5, 6], and each responds differently to various environmental signals [1, 7]. In addition, the catalase isozymes exhibit cell and organelle specificities [1, and references therein]. For example, CAT-2 first appears during late kernel development and increases dramatically in the scutellum after germination. CAT-2 is absent in etiolated leaves, but rapidly accumulates upon exposure to light due to increased transcript accumulation and translation of the *Cat2* message.

The nucleotide sequence data reported will appear in the EMBL Nucleotide Sequence Database under the accession number Z54358.

Thus, unlike *Cat1* which does not respond to light and *Cat3* which is controlled by a circadian clock, *Cat2* is positively regulated by light in a tissue-specific manner [1, 8].

In order to understand the underlying mechanisms by which the *Cat* genes are regulated and expressed in response to various signals, their cDNAs were isolated and used, in turn, to isolate the respective genes. The *Cat1* and *Cat3* genes were successfully isolated from a genomic library and fully characterized [9, 10]. The *Cat2* gene was recently cloned by two different methods: direct cloning and PCR. Herein, we report on the isolation, characterization, and expression of the *Cat2* gene, and its comparison to the other maize catalase genes.

Materials and methods

Isolation and purification of digested genomic DNA

W64A genomic DNA was isolated from 14-day-old light grown leaves as described [11]. A large amount of total DNA (50 μg) was digested with the restriction enzymes *Bam*HI and *Xba*I and electrophoresed on a 0.4% agarose gel. Agarose slices containing DNA fragments between 2.0 to 4.0 kb in size were sliced into 6 agarose pieces. DNA purification was conducted by a special freeze-thaw method. The procedure proved to be an effective and efficient method in comparison to other commercially available DNA purification kits. This procedure is fast, inexpensive, and results in high-quality DNA ready for labeling and library construction. It can be effectively used for both low- and high-molecular-weight DNA purification. The detailed procedure is as follows: agarose slices were transferred into a microfuge tube and were quickly frozen in liquid N_2. Agarose slices were quickly thawed at 50 °C for 10 min. The tube was then centrifuged for 5 min and the aqueous solution containing eluted DNA was transferred into a new tube. The tube containing sliced agarose was frozen and thawed again to elute more DNA solution and the newly eluted liquid was transferred and combined with the previous eluted DNA. The efficiency of the DNA elution can be visualized under a UV light box. Phenol extraction was conducted to obtain pure DNA for genomic library construction. Ethidium bromide was removed from DNA by ethanol precipitation.

Preparation of the plasmid vector for genomic library construction

Because of the relatively small size of the insert DNA, we used plasmid pBluescript KS (−) as the vector for library construction. This plasmid was digested with the restriction enzymes *Xba*I/*Bam*HI and was purified by phenol/chloroform extraction.

Ligation and transformation

Genomic DNA fragments containing the *Cat2* 5′ sequence were ligated into the *Bam*HI/*Xba*I-digested pBluescript vector. Ligation was set at 1:1 and 1:2 vector-to-insert ratio. Ligated plasmids were transformed into the XL-1 Blue supercompetent cells according to the manufacturer's instructions (Stratagene). Transformation efficiency was 2×10^7 cfu/μg vector with supercompetent cells and 1:2 vector-to-insert ratio. The library was screened with a 5′ end *Cat2* cDNA fragment and positive clones were identified.

PCR primers

Two 21-mer primers for the genomic DNA PCR amplification reaction were synthesized (NCSU Molecular Genetics Facility). The 5′ primer begins at the 22nd nucleotide of the known first *Cat2* intron (see Results for details) and its sequence is 5′-GTCTCAATTCGTGTTTCGTCG-3′. The 3′ primer was selected to span 11 nucleotides in the 3′-untranslated region and the last 10 translated nucleotides of the *Cat2* cDNA. The 3′ primer sequence is: 5′-GGTTGATCTTACATGCTCGGC-3′. Control PCR amplifica-

tion of the translated sequence of the *Cat2* cDNA was performed with plasmid p*Cat2*.1c template [12], the same 3′ primer as in the genomic PCR and a 19-mer 5′ primer from the start of translation with sequence 5′-CCATGGACCCGTACAAGCA-3′.

Polymerase chain reaction

PCR was performed in a MJ Research PTC-100 programmable thermal controller, using either *Thermus aquaticus* DNA polymerase (*Taq* DNA polymerase; Stratagene) or *Thermoccocus litoralis* DNA polymerase (Vent; New England Biolabs). The reaction mixture, in a total volume of 100 μl, contained, when using *Taq* polymerase: 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml nuclease-free BSA, 200 μM dNTPs, 1 μM of each primer, 5 units *Taq* Extender (Stratagene), 2.5 units *Taq* polymerase, and 0.5 μg template genomic DNA or 10 ng template plasmid DNA. The reaction mixture, when using Vent polymerase, was the same with the exceptions that the BSA and the *Taq* Extender were omitted and instead of *Taq*, 1 unit of Vent polymerase was used. The cycle program was, 3.5 min denaturation at 95 °C followed by 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 60 °C, and 1.5 min extension at 75 °C. The time of the last extension step was increased at 6 min to ensure that all the PCR products were complete double-stranded blunt-ended fragments. No differences were observed in the PCR products after the use of either Vent or *Taq* polymerases. Vent polymerase amplified DNA was used in further experiments, because Vent also contains a 3′-5′ proofreading exonuclease activity resulting in higher fidelity of base incorporation compared to *Taq* polymerase, which lacks this function.

Purification and cloning of the PCR product

The PCR products were analyzed by agarose gel electrophoresis and the specific *Cat2* band was purified and directly cloned into the *Sma*I site of the pBluescript II KS(−) (Stratagene). That the PCR product was in fact *Cat2* was verified by hybridization with the *Cat2* cDNA probe.

Sequencing

The DNA sequence of the *Cat2* 5′ and the coding region clones were determined by the dideoxy nucleotide chain termination method [13]. Overlapping deletions for sequencing were generated using ExoIII nuclease on double-stranded plasmid templates [14]. Template plasmids were isolated and purified by the Jetprep plasmid DNA isolation and purification kit (Genomed, Research Triangle Park, NC). Sequencing was performed by the Applied Biosystems Automated DNA Sequencer (Nucleic Acid Facility, Iowa State University).

Primer extension

Primer extension was used to determine the start site of transcription of *Cat2*. The procedure was according to Metraux *et al.* [15]. A 22-mer oligonucleotide primer (5′-TTGTACGGGTCCATGGCGGTGG-3′) was synthesized (NCSU Molecular Genetics Facility) which is complementary to the 5′ end of the *Cat2* cDNA. This primer was end-labeled with polynucleotide kinase. A 50 μg portion of total RNA (isolated from salicylic acid (SA)-treated scutella with increased *Cat2* transcript, 28 days after pollination [7]) was obtained. The modified procedure was described [9]. Extension products were electrophoresed on an 8% sequencing gel with sequencing reaction products of the *Cat2* promoter fragment using the same oligo primer.

Hormone treatment and RNA analysis

Maize W64A embryos were manually excised from germinating seed at 2 and 5 days post imbibition (dpi). Excised embryos were incubated

on MS basic salt plates [16] supplemented with 5 mM/10 mM of gibberellin (GA_3), 100 mM abscisic acid (ABA) (5 dpi), and 1 mM/1.5 mM salicylic acid (2 dpi) for 24 h in the dark. After treatment, scutella were harvested, frozen in liquid nitrogen and stored at −70 °C. Total RNA was isolated from treated scutella by cold phenol extraction [17]. Total RNA (20 μg) from each sample was separated on denaturing 1.2% agarose gels, and transferred to either nitrocellulose or nylon membranes. The resulting blots were hybridized with ^{32}P-labeled *Cat2* gene-specific probe. After this analysis was performed, the probe was removed from the filters by repeated washes in boiling 0.1% SSC, 0.1% SDS. Equal sample loading was verified by reprobing the filters with a cloned fragment containing 18S rDNA [18].

Results

Isolation and characterization of the 5′ end of the Cat2 *gene by direct cloning*

The *Cat1* and *Cat3* genes of maize were previously isolated from a W64A genomic library and fully characterized [9, 10]. The maize *Cat2* gene could not be isolated from the same library. We attempted a different approach in order to isolate the *Cat2* genomic clone. We have tested the possibility of isolating a partial genomic clone containing the 5′ promoter region which is important for *Cat2* regulation by completely digesting with restriction enzymes. Preliminary results from Southern blot analysis indicated that a 2.3 kb *Xba*I/*Bam*HI genomic DNA fragment contained the 5′ portion of the *Cat2* coding and 5′ promoter regions. Genomic DNA was completely digested with *Xba*I/*Bam*HI and about 2.3 kb of this DNA was isolated and purified (Fig. 1A). Purified genomic DNA fragments were electrophoresed on a 1% agarose gel, transferred onto a nitrocellulose filter and probed with a *Cat2* 5′ end cDNA fragment; one positive fraction was used for genomic library construction (Fig. 1B).

About 50000 colonies were screened with the

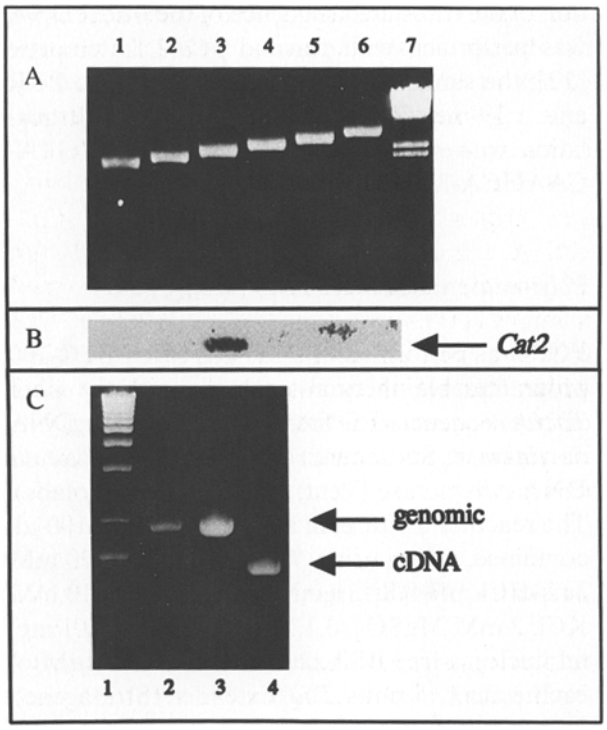


Fig. 1. W64A genomic DNA fragments purified by the freeze-thaw method. A. W64A genomic DNA (50 μg) was digested with *Bam*HI/*Xba*I and electrophoresed on a 0.4% agarose gel. Digested DNA fragments sized from 2.3 to 4 kb were sliced into 6 gel pieces and purified from agarose gels as described in Materials and methods. Fractions of purified DNA fragments were electrophoresed on a 1% agarose gel as indicated in lanes 1 to 6. Lane 7 is lambda *Hin*dIII DNA marker. B. The same DNA gel was transferred onto a nitrocellulose membrane and probed with a 5′ *Cat2* cDNA fragment (*Eco*RI/*Bam*HI). Results indicate that lane 3 contains most of the *Cat2* genomic DNA fragments and was used for genomic library construction. C. Comparison of the PCR products using different *Cat2* templates. Lane 1. 1 kb ladder (BRL) molecular weight markers. Lane 2, PCR amplification with Vent polymerase and 0.5 μg of maize genomic DNA as template. Lane 3, PCR amplification of *Cat2* genomic coding region using 10 ng of lane 2 PCR product DNA as template. Lane 4, PCR amplified *Cat2* cDNA using 10 ng of lane 2 PCR product DNA as template. Lane 4, PCR amplified *Cat2* cDNA using 10 ng of p*Cat2*.1c plasmid as template. The *Cat2* genomic bands and the *Cat2* cDNA band are indicated by arrows. An apparent difference, ca. 400 bp in size can be observed suggesting the existance of intron(s) in the *Cat2* genomic coding region.

Cat2 5′ end cDNA probe. Ten positive clones were identified and re-screened to eliminate any possible false positives. The plasmid DNA was

isolated in order to identify the proper insertion. Southern blots were used to confirm the positive clones and the restriction map was defined by digesting all positive clones with various restriction enzymes. Results indicated that all ten positive clones were identical. One of these clones was sequenced. On comparison to the *Cat2* cDNA, this clone was found to contain a 1.6 kb 5′ promoter sequence. The coding region ranged from the ATG start codon to the first *Bam*HI site (600 bp) (Fig. 2). Two introns were identified within that region by comparison with the *Cat2* cDNA sequence. The first intron (92 bp long) was identified at five amino acids from the start of translation.

Isolation of the Cat2 *gene coding region by PCR*

In preliminary experiments, the primers designed for the *Cat2* cDNA amplification were used in an attempt to amplify the *Cat2* genomic coding region, with no success. After the genomic clone corresponding to the 5′ of the *Cat2* coding region was isolated and sequenced, a 92 bp intron occurring 15 nucleotides from the first ATG codon was revealed. The last three nucleotides of the 5′ cDNA primer were mismatching to the genomic DNA sequence due to the occurrence of that intron. Thus, a new 5′ primer from the intron sequence was designed, to ensure that any PCR amplified sequence should represent *Cat2* genomic DNA. A single strong band about 1900 bp was observed after amplification of genomic DNA with the Vent polymerase. The 1900 bp band was excised from the gel to avoid any minor contamination and the recovered DNA was directly ligated into the *Sma*I site of the pBluescript vector. Plasmid DNA from clones with the correct insert served as template for PCR amplification to ensure that the insert is *Cat2* genomic DNA. *Cat2* cDNA from the plasmid p*Cat2*.1c [12] was also amplified using the *Cat2* cDNA designed primers. The amplified *Cat2* genomic coding region migrated as a 1900 bp band from both templates used. The *Cat2* cDNA formed a 1480 bp band, as was expected. The apparent dissimilarity between the *Cat2* genomic coding region and the *Cat2* cDNA band suggested that one or more introns was responsible for the about 400 bp difference in length of the two bands (Fig. 1C). In order to gain more information about the number and possibly the location(s) as well as the orientation of the insert in one clone selected for further analysis, restriction digestion with several enzymes and sequencing were performed. Both indicated that in addition to the first intron which was used for making the 5′ primer, four more introns were present. Comparison of the two clones isolated by direct cloning and by PCR as well as the *Cat2* cDNA revealed no mismatches in common regions.

Structure and expression of the maize Cat2 *gene*

Sequencing results showed that the overlapping regions of the two clones have identical sequences, indicating that both clones are part of the same gene. Previous results indicated that only one

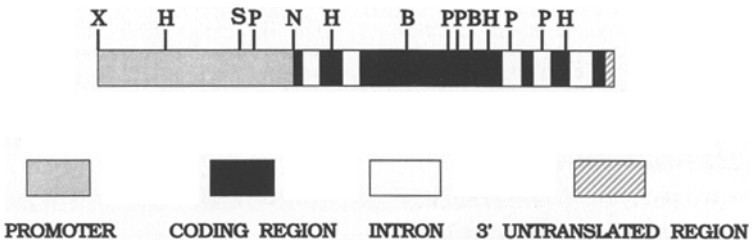
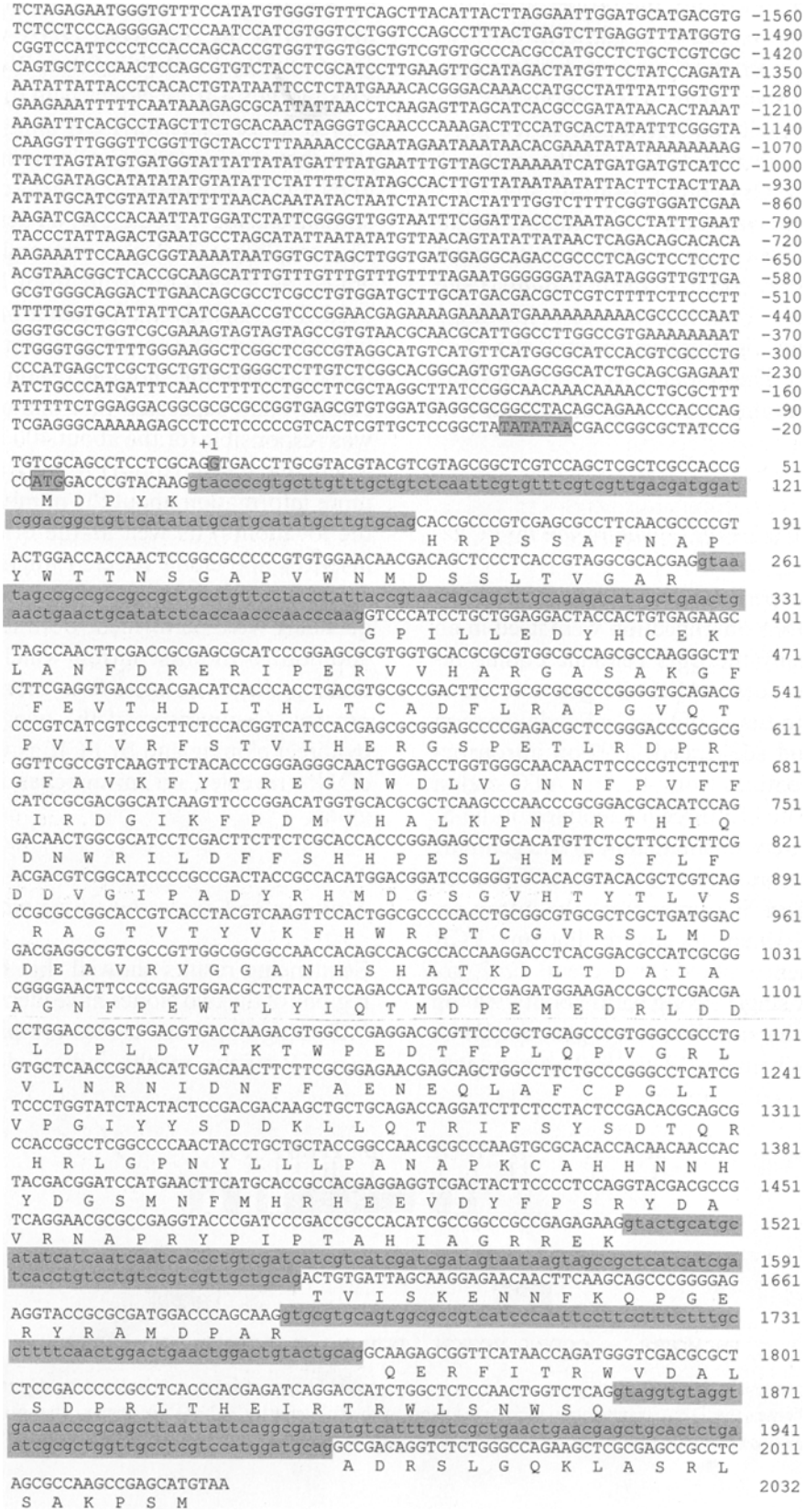


Fig. 2. Restriction map of the maize *Cat2* gene. The map was constructed from two clones isolated by direct cloning and PCR. The black box indicates the DNA fragments which hybridized to the full length *Cat2* cDNA probe. The introns, coding region, and 5′- and 3′-flanking regions are indicated. Restriction sites are: B, *Bam*HI; H, *Hin*dII; N, *Nco*I; P, *Pst*I; S, *Sac*I; X, *Xba*I.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TCTCCTCCCAGGGGACTCCAATCCATCGTGGTCCTGGTCCAGCCTTTACTGAGTCTTGAGGTTTATGGTG -1490
CGGTCCATTCCCTCCACCAGCACCGTGGTTGGTGGCTGTCGTGTGCCCACGCCATGCCTCTGCTCGTCGC -1420
CAGTGCTCCCAACTCCAGCGTGTCTACCTCGCATCCTTGAAGTTGCATAGACTATGTTCCTATCCAGATA -1350
AATATTATTACCTCACACTGTATAATTCCTCTATGAAACACGGGACAAACCATGCCTATTTATTGGTGTT -1280
GAAGAAATTTTTCAATAAAGAGCGCATTATTAACCTCAAGAGTTAGCATCACGCCGATATAACACTAAAT -1210
AAGATTTCACGCCTAGCTTCTGCACAACTAGGGTGCAACCCAAAGACTTCCATGCACTATATTTCGGGTA -1140
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ACGTAACGGCTCACCGCAAGCATTTGTTTGTTTGTTTGTTTTAGAATGGGGGGATAGATAGGGTTGTTGA -580
GCGTGGGCAGGACTTGAACAGCGCCTCGCCTGTGGATGCTTGCATGACGACGCTCGTCTTTTCTTCCCTT -510
TTTTTGGTGCATTATTCATCGAACCGTCCCGGAACGAGAAAAGAAAAATGAAAAAAAAAACGCCCCCAAT -440
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CTGGGTGGCTTTTGGGAAGGCTCGGCTCGCCGTAGGCATGTCATGTTCATGGCGCATCCACGTCGCCCTG -300
CCCATGAGCTCGCTGCTGTGCTGGGCTCTTGTCTCGGCACGGCAGTGTGAGCGGCATCTGCAGCGAGAAT -230
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TTTTTTCTGGAGGACGGCGCGCGCCGGTGAGCGTGTGGATGAGGCGCGGCCTACAGCAGAACCCACCCAG -90
TCGAGGGCAAAAAGAGCCTCCTCCCCCGTCACTCGTTGCTCCGGCTATATATAACGACCGGCGCTATCCG -20
+1
TGTCGCAGCCTCCTCGCAGGTGACCTTGCGTACGTACGTCGTAGCGGCTCGTCCAGCTCGCTCGCCACCG 51
CCATGGACCCGTACAAGgtaccccgtgcttgtttgctgtctcaattcgtgtttcgtcgttgacgatggat 121
M D P Y K
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H R P S S A F N A P
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Y W T T N S G A P V W N M D S S L T V G A R
tagccgccgccgccgctgcctgttcctacctattaccgtaacagcagcttgcagagacatagctgaactg 331
aactgaactgcatatctcaccaacccaacccaagGTCCCATCCTGCTGGAGGACTACCACTGTGAGAAGC 401
G P I L L E D Y H C E K
TAGCCAACTTCGACCGCGAGCGCATCCCGGAGCGCGTGGTGCACGCGCGTGGCGCCAGCGCCAAGGGCTT 471
L A N F D R E R I P E R V V H A R G A S A K G F
CTTCGAGGTGACCCACGACATCACCCACCTGACGTGCGCCGACTTCCTGCGCGCGCCCGGGGTGCAGACG 541
F E V T H D I T H L T C A D F L R A P G V Q T
CCCGTCATCGTCCGCTTCTCCACGGTCATCCACGAGCGCGGGAGCCCCGAGACGCTCCGGGACCCGCGCG 611
P V I V R F S T V I H E R G S P E T L R D P R
GGTTCGCCGTCAAGTTCTACACCCGGGAGGGCAACTGGGACCTGGTGGGCAACAACTTCCCCGTCTTCTT 681
G F A V K F Y T R E G N W D L V G N N F P V F F
CATCCGCGACGGCATCAAGTTCCCGGACATGGTGCACGCGCTCAAGCCCAACCCGCGGACGCACATCCAG 751
I R D G I K F P D M V H A L K P N P R T H I Q
GACAACTGGCGCATCCTCGACTTCTTCTCGCACCACCCGGAGAGCCTGCACATGTTCTCCTTCCTCTTCG 821
D N W R I L D F F S H H P E S L H M F S F L F
ACGACGTCGGCATCCCCGCCGACTACCGCCACATGGACGGATCCGGGGTGCACACGTACACGCTCGTCAG 891
D D V G I P A D Y R H M D G S G V H T Y T L V S
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R A G T V T Y V K F H W R P T C G V R S L M D
GACGAGGCCGTCGCCGTTGGCGGCGCCAACCACAGCCACGCCACCAAGGACCTCACGGACGCCATCGCGG 1031
D E A V R V G G A N H S H A T K D L T D A I A
CGGGGAACTTCCCCGAGTGGACGCTCTACATCCAGACCATGGACCCCGAGATGGAAGACCGCCTCGACGA 1101
A G N F P E W T L Y I Q T M D P E M E D R L D D
CCTGGACCCGCTGGACGTGACCAAGACGTGGCCCGAGGACGCGTTCCCGCTGCAGCCCGTGGGCCGCCTG 1171
L D P L D V T K T W P E D T F P L Q P V G R L
GTGCTCAACCGCAACATCGACAACTTCTTCGGCGGAGAACGAGCAGCTGGCCTTCTGCCCGGGCCTCATCG 1241
V L N R N I D N F F A E N E Q L A F C P G L I
TCCCTGGTATCTACTACTCCGACGACAAGCTGCTGCAGACCAGGATCTTCTCCTACTCCGACACGCAGCG 1311
V P G I Y Y S D D K L L Q T R I F S Y S D T Q R
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H R L G P N Y L L L P A N A P K C A H H N N H
TACGACGGATCCATGAACTTCATGCACCGCCACGAGGAGGTCGACTACTTCCCCTCCAGGTACGACGCCG 1451
Y D G S M N F M H R H E E V D Y F P S R Y D A
TCAGGAACGCGCCGAGGTACCCGATCCCGACCGCCCACATCGCCGGCCGCCGAGAGAAGgtactgcatgc 1521
V R N A P R Y P I P T A H I A G R R E K
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tcacctgtcctgtccgtcgttgctgcagACTGTGATTAGCAAGGAGAACAACTTCAAGCAGCCCGGGGAG 1661
T V I S K E N N F K Q P G E
AGGTACCGCGCGATGGACCCAGCAAGgtgcgtgcagtggcgccgtcatcccaattccttcctttctttgc 1731
R Y R A M D P A R
cttttcaactggactgaactggactgtactgcagGCAAGAGCGGTTCATAACCAGATGGGTCGACGCGCT 1801
Q E R F I T R W V D A L
CTCCGACCCCCGCCTCACCCACGAGATCAGGACCATCTGGCTCTCCAACTGGTCTCAGgtaggtgtaggt 1871
S D P R L T H E I R T R W L S N W S Q
gacaacccgcagcttaattattcaggcgatgatgtcatttgctcgctgaactgaacgagctgcactctga 1941
atcgcgctggttgcctcgtccatggatgcagGCCGACAGGTCTCTGGGCCAGAAGCTCGCGAGCCGCCTC 2011
A D R S L G Q K L A S R L
AGCGCCAAGCCGAGCATGTAA 2032
S A K P S M

copy of the *Cat2* gene exists in the maize genome [11]. A restriction map (Fig. 2) was constructed based on a comparison of the *Cat2* cDNA sequence with the *Cat2* gene sequence (Fig. 3).

In order to map the 5′ end of the *Cat2* transcript, primer extension was performed on total RNA isolated from 1 mM SA-treated scutella [7] 28 days after pollination in which the *Cat2* transcript was increased to high levels. The *Cat2* cDNA contains a short 5′-untranslated region. In order to obtain a fair size of extension product, a 22-mer oligonucleotide was synthesized which was complementary to both *Cat2* cDNA and genomic DNA. It covered the portion of 5′-untranslated region and coding region up to the first intron. The primer extension product is about 45 bp and the 5′ end is indicated as + 1 in Fig. 3. Upstream from the transcriptional start site, several motifs were found and are depicted in Fig. 4. A typical TATA box is located at −43. The CAAT consensus sequence cannot be found between −80 and −120; however, two sequence motifs (GCGCGG, GGGCAG), which are homologous to the consensus sequences for the Sp1-binding site, GGGCGG (GC box) [19] were found around −114 and −570. The GC box has been found in promoters of many viral and cellular genes [20], and acts as a binding site of a protein, Sp1, which is necessary for transcriptional activity. A pyrimidine box (CCTTT) and Box I (GCAGTG) which are part of the GA response complex [21] were found at −208 and −256. Two 8 bp sequences (CACGTCGC, CACGTAAC) which are similar to an ABA response element (ABRE, CACGTGGC) [22] were located at −308, −648 relative to the + 1 site. The core sequence of the ABA response element (ACGT) is the binding site for basic leucine zipper transcriptional factors or common plant regulatory factors (CPRFs) [23]. Promoter elements with a ACGT core are recognized by both homo- and heterodimers of leucine zipper transcriptional factors. A CE1-like element which was recently reported to enhance the ABA response [24], was located next to the second ABRE at −636 (Fig. 4). A 6 bp inverted repeat sequence (CGGCGC,GCGCCG) was also found. An 11 bp element (GGTGACCTTGC), which is identical to the antioxidant response element (ARE, PuGTGACNNNGC) of the rat glutathione *S*-transferase Ya subunit [25], was identified at a position close to the + 1 site. The ARE might represent a *cis*-acting element which activates genes that protect eukaryotic cells against oxidative stress. The ARE was also found at −470 of the maize *Cat1* promoter [9]. Two direct ACGT repeat sequences, which is the core sequence for leucine zipper protein binding sites, were located at about 15 bp downstream of the + 1 site. A DNA motif CAGGTG, which is identical to the core sequence for a class of transcriptional factors bHLH (CANNTG, basic helix-loop-helix proteins), was located near the + 1 site and overlapping with the 5′ portion of the ARE motif. This transcriptional factor shares similarities with the basic leucine zipper family and can form homo- and heterodimers to exert regulatory functions [26]. Two additional bHLH binding motifs (CATCTG, CATTTG) were also found at −245, and −625 relative to the + 1 site. Both of them are located down stream of the two leucine zipper core sequences ACGT. A DNA sequence (CGTCCCGGAACG) was located at −480 with 2 bp mismatch to the 12 bp metal responsive element (MRE, CGNCCCGGNCNC) core sequence [27]. This motif can be found in the promoter region of metallothionein proteins that bind heavy metal ions, and have been functionally implicated in heavy metal detoxification [28]. The putative regulatory motifs found in the 5′ of the *Cat2* gene are summarized in Fig. 5.

The DNA coding sequence in the coding region of the *Cat2* genomic DNA is identical to the cDNA. The map and sequence of the *Cat2* gene

Fig. 3. Nucleotide and deduced amino acid sequence of the maize *Cat2* gene. The *Cat2* clones were sequenced by the dideoxynucleotide chain termination method. The deduced amino acid sequence is shown in single letter code below the nucleotide sequence. Introns are shown in lower-case letters. The transcriptional start site was determined by primer extension and is indicated as + 1. The translational codon (ATG) and TATA box are also indicated.

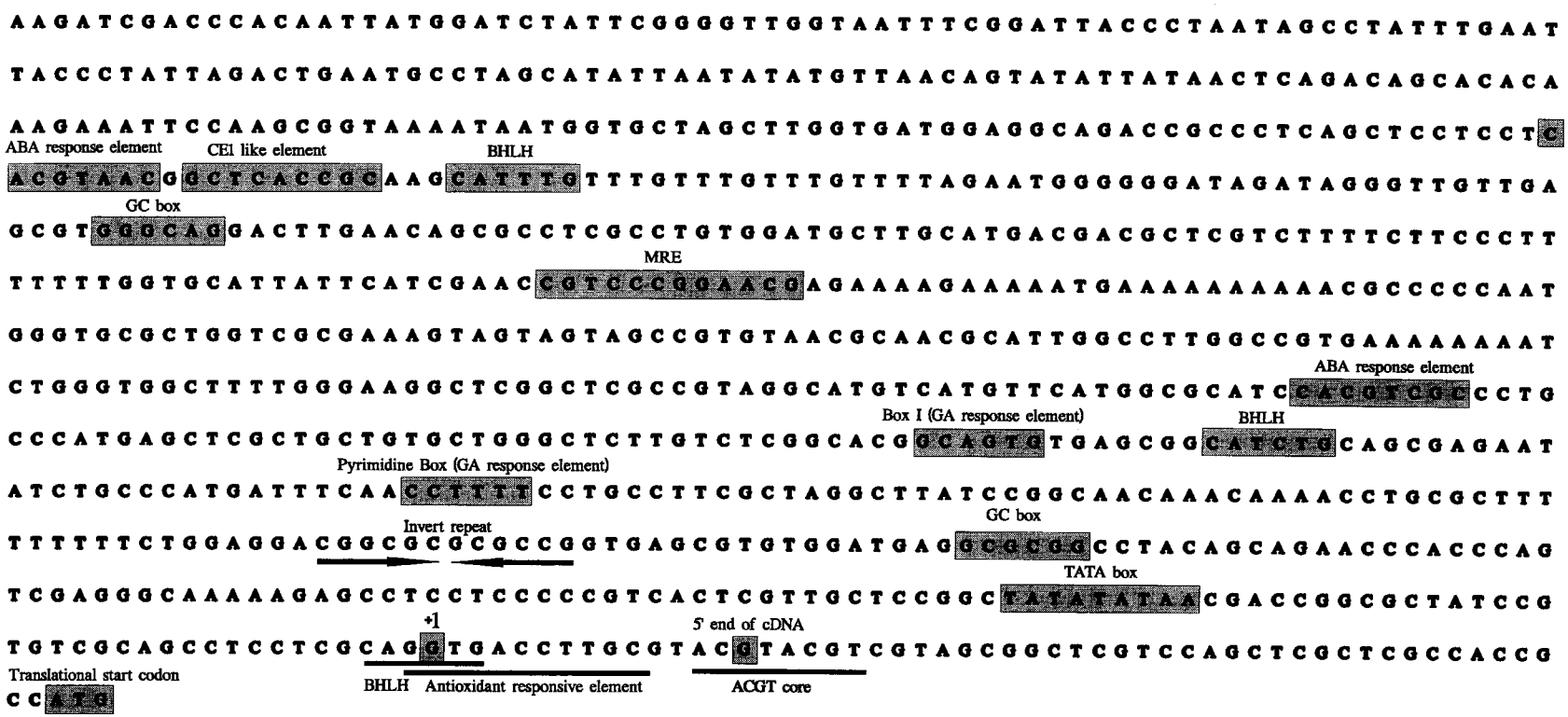

Fig. 4. Nucleotide sequence of the maize *Cat2* genomic DNA 5′-flanking region. This region extends from −860 (relative to the start of transcription + 1) to + 56, the translational start codon. The ATG codon, 5′ end of the cDNA and + 1 site are indicated. Arrows indicate the direct repeat sequence. Some important motifs for plant gene regulation are also indicated and boxed, including TATA box, two GC boxes, two ABRE, CE1 like elements, metal responsive element (MRE), antioxidant-responsive element (ARE), three BHLH (basic helix-loop-helix), and part of the GA response element (pyrimidine box and box I).

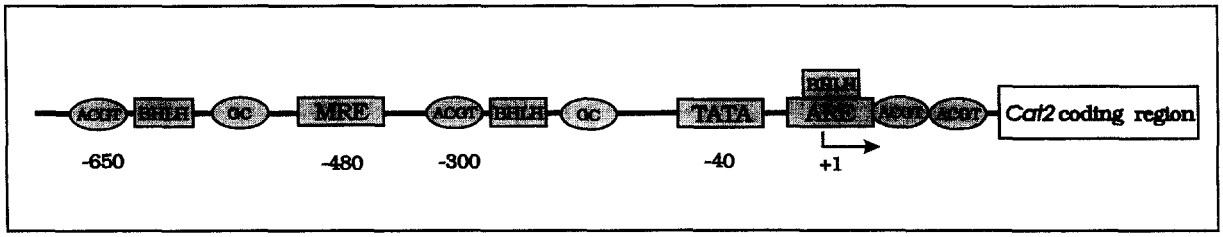

Fig. 5. Schematic representation of the putative motifs located in the promoter region of the *Cat2* gene. The abbreviations are: BHLH, basic helix-loop-helix protein binding site; ARE, antioxidant-responsive element; ACGT, ACGT core or leucine zipper protein binding site; GC, GC box or Sp1-binding site; MRE, metal-responsive element.

indicated that the coding region contains five short introns. All introns possess the consensus 5′ GT splice donor site and the 3′ AG splice acceptor site described in other eukaryotic genes [29]. The properties of these five introns are indicated in Fig. 6. Intron sizes are relatively small ranging from 78 to 113 bp. The sequence at the 5′ and 3′ splice sites of the introns have low homologies to the consensus sequence (CAG/GTAAGT and TGCAG/GT) for plant introns [30]. In addition, all five introns are atypical from other plant introns in that they are not AT-rich. All five *Cat2* introns are located in exactly the same positions as five of the six introns in *Cat1*. The number three intron in *Cat1* is missing in the *Cat2* gene (Fig. 7). In contrast to the *Cat1* introns, all *Cat2* introns are about 50% AT nucleotides and their sizes are relatively small in comparison to the *Cat1* introns. Introns 1 and 5 of the *Cat2* gene are located at the same position as the two introns in the *Cat3* gene. These results indicate evolutionary linkages among the three maize catalase genes.

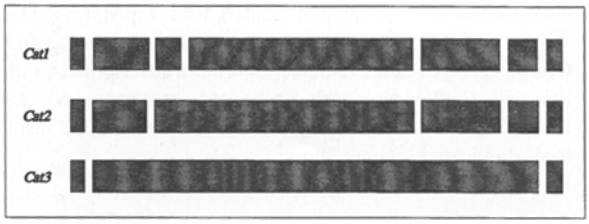

Fig. 7. Exon/intron structure of the three maize catalase genes. Exons (dark boxes) and introns (gaps) are drawn schematically to indicate the relative position of the introns. The maize *Cat1* gene [9] possesses 6 introns, the *Cat2* gene (this study) has 5 introns, and *Cat3* contains only 2 introns [10].

The expression of the maize *Cat2* gene is highly regulated developmentally and spatially [1]. The *Cat2* transcript can be detected during the late stages of seed development. Upon germination, the *Cat2* transcript increases dramatically and reaches a peak at about 4 days after imbibition. The responses of *Cat2* to several environmental factors have also been examined. *Cat2* responds to the fungal toxin cercosporin differently at two distinct developmental stages: embryogenesis and

Properties of *Cat2* gene introns

Intron #	Junction sequence	Size (bp)	A+T (%)
1	CAAG/GTACCC.....TGCAG/CA	92	51
2	CGAG/GTAATA.....CCAAG/GT	108	47
3	GAAG/GTACTG.....TGCAG/AC	109	52
4	CAAG/GTGCGT.....TGCAG/GC	78	47
5	TCAG/GTAGGT.....TGCAG/GC	113	49

Fig. 6. Properties of the maize *Cat2* introns. The intron/exon junction sequences were located by comparison with the *Cat2* cDNA sequence. The size of *Cat2* introns varies from 78 to 113 bp.

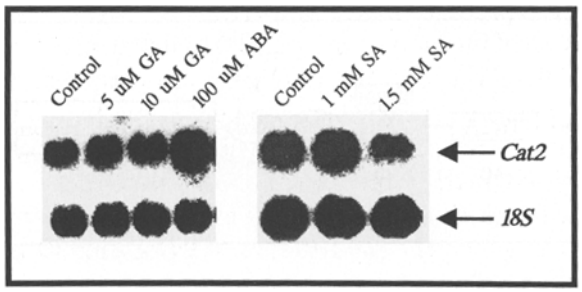

Fig. 8. Responses of the maize *Cat2* gene to GA_3, ABA and SA in mature maize embryos. W64A embryos were excised from 5 dpi seeds and treated with 5 mM, 10 mM of gibberellic acid (GA_3), 100 mM of abscisic acid (ABA); embryos were also excised from 2 dpi and treated with 1 mM/1.5 mM of salicylic acid (SA) as described. Scutella were isolated from treated embryos and examined for *Cat2* transcript accumulation with *Cat2* gene-specific probe and reprobed with a 18S DNA fragment as a loading control. Representative results from duplicate blots are shown.

germination [31]. The *Cat2* gene also responds to SA differentially at these two stages [7]. In order to gain a better understanding of the developmental stage-dependent responses of *Cat2*, we extended our studies to examine the response of *Cat2* to plant growth regulators at later stages of germination. Germinating embryos were treated with GA, ABA at 5 dpi and SA at 2 dpi for 24 h. Northern blots were then performed with a *Cat2* gene-specific fragment. Results indicated that the *Cat2* transcript did not change upon GA treatment; however, the *Cat2* transcript from scutella 5 days after imbibition increased dramatically following ABA treatment, and *Cat2* transcript increased slightly after 1 mM SA treatment at 2 dpi (Fig. 8).

Discussion

The maize *Cat2* genomic DNA was isolated by direct cloning and PCR. A 2.3 bp genomic clone containing the 5′ end of the *Cat2* gene was isolated by the direct cloning method. The fragment includes 1.7 kb of sequence 5′ of the *Cat2* gene, two small introns shortly after the ATG start codon, and 600 bp of coding sequence. The small intron located 5 amino acids from the amino terminus is located exactly at the same position as the first introns of the *Cat1* and *Cat3* genes. No sequence similarity was found among the first introns of the three maize *Cat* genes. The coding region of the *Cat2* gene was cloned and isolated by PCR with the primer designed from the first intron. The resulting PCR product shared about 400 bp identical with the 3′ portion of the 2.3 kb clone. This indicated that both clones are part of the same *Cat2* gene. Previous DNA blot data also suggested that only one copy of the *Cat2* gene exists in the maize genome [11]. In the coding region of the *Cat2* gene, 5 introns were located and their locations are exactly the same as five of the maize *Cat1* introns. They also share the same locations with five castor bean introns [32]. The identical positions of the catalase introns between the monocot and dicot plants imply evolutionary links among plant catalases.

The *Cat2* gene promoter region revealed an obvious TATA box located 43 bp 5′ from the end of the cDNA. No CAAT box was found within 120 bp upstream from the 5′ end *Cat2* cDNA; however, several motifs involved in plant gene regulation were identified. Two putative ABA response elements (CACGTCGC, CACGTAAC) were identified in the *Cat2* promoter region. One of the elements (CACGTCGC, −308) is almost identical to the ABA response element (CACGTGGC) of the wheat *Em* gene [22]. However, the *Cat2* gene responds negatively to ABA in scutella after pollination and the *Cat2* transcript failed to accumulate after 24 h of ABA treatment [33]. In this report, we found that the *Cat2* transcript increased after 24 h ABA treatment at 5 dpi, suggesting that the response of the *Cat2* gene to ABA is developmental stage specific. Whether the effect is direct or indirect still needs to be determined. This ABA response element contains the core sequence ACGT which is a *cis*-acting element for plant transcription factors. It is also the core element for response to light [34, 35], jasmonic acid [36], and salicylic acid [37]. The *Cat2* gene responds positively to light [8] and salicylic acid [7]. It is possible that the ACGT core sequence in the *Cat2* gene also serves as a light or SA response element. At 1 mM

of SA concentration, *Cat2* transcript increased dramatically in 28 dpp scutella; however, the *Cat2* transcript failed to accumulate at 1 dpi with the same doses of SA treatment [7]. We extended our study to examine the effect of SA on *Cat2* at 2 dpi. Surprisingly, the *Cat2* transcript again increased slightly upon 1 mM SA treatment. The levels of transcript accumulation are not as dramatic as they are during embryogensis. Thus, the response of *Cat2* to the same doses of SA is not only different at two distinct developmental stages (i.e., embryogenesis and germination), but also differ at two time points of the same developmental stage. A pyrimidine box (CCTTTT) and box I (GCAGTG) of the GA response complex of a barley α-amylase [21] were also identified. Recently, new evidence indicated that the *Cat2* gene is induced by a germination related regulator [7]. This regulator might be GA or some other plant hormone. To address this question, maize embryos from 5 dpi were isolated and incubated in culture plates containing GA, and the *Cat2* transcript level was examined by northern blots. Results showed that there are no changes in *Cat2* transcript levels between GA treated and non-treated maize embryos at 5 dpi. This is also the case with 28 dpp embryos (data not shown). These results suggest that GA is not the regulatory factor which induces *Cat2* expression upon germination.

Several regulatory motifs were found in the area around the transcription start site and 5′-untranslated region as well. Two repeat ACGT core sequences were located down stream of the + 1 site. An antioxidant responsive element and a bHLH binding site were located around the + 1 site. The function of these putative motifs at the + 1 and 5′-untranslated region still needs to be determined. It may be that they are involved in post-transcriptional mechanisms which regulate the *Cat2* gene. Further upstream in the *Cat2* promoter, two other bHLH motifs were also located near two ACGT core sequences. The same pattern was observed in the 5′ region of the maize *Cat1* gene (two bHLH located near two ACGT core sequences) [9]. The role of the sequential presence of these elements in the two catalase gene promoters is being investigated. The expression of the maize *Cat2* gene is regulated at several different levels. The *Cat2* gene also responds to signals at specific developmental stages. Thus, tissue and stage specific regulatory factors might be involved in the regulation of *Cat2* gene. Further experiments are underway to identify the *cis*-acting elements and the *trans*-acting factors responsible for *Cat2* expression.

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