

# The Maize (*Zea mays* L.) *Cat1* Catalase Promoter Displays Differential Binding of Nuclear Proteins Isolated from Germinated and Developing Embryos and from Embryos Grown in the Presence and Absence of Abscisic Acid<sup>1</sup>

John D. Williamson<sup>2</sup> and John G. Scandalios\*

Department of Genetics, Box 7614, North Carolina State University, Raleigh, North Carolina 27695–7614

We previously demonstrated that amounts of *Cat1* RNA in developing immature maize (*Zea mays* L.) embryos change in parallel with endogenous abscisic acid (ABA) content. In excised immature embryos, addition of ABA leads to a large increase in *Cat1* RNA accumulation. The *Cat1* transcript, however, also accumulates to high amounts in scutella of germinating embryos, where ABA content is low and decreasing. Here we show that application of ABA to germinated embryos no longer results in the up-regulation of the *Cat1* transcript accumulation that is seen during embryogenesis. This suggests that factors other than ABA control *Cat1* expression at this developmental stage. Using band-shift and southwestern analyses, we show that the change in sensitivity to ABA is paralleled by changes in nuclear proteins binding to a 28-bp region of the *Cat1* promoter in vitro. One protein (CAT1BP-20) shows increased accumulation in the absence of ABA, suggesting that a repressor-mediated mechanism accounts for at least a portion of the ABA regulation of *Cat1*.

Catalase (H<sub>2</sub>O<sub>2</sub>:H<sub>2</sub>O<sub>2</sub> oxidoreductase, EC 1.11.1.6; CAT) is a tetrameric, heme-containing enzyme that catalyzes the dismutation of H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O and O<sub>2</sub>. In maize (*Zea mays* L.) three unlinked structural genes, *Cat1*, *Cat2*, and *Cat3*, encode the biochemically distinct catalase isozymes, CAT-1, CAT-2, and CAT-3. The monomeric subunits are each approximately 60 kD in size and are structurally similar to catalase subunits from other organisms (Scandalios, 1965, 1990; Scandalios et al., 1980). The differential spatial and temporal expression of the three CAT isozymes has been extensively studied in maize. The present study focuses on the expression of maize CAT-1, the major catalase in the scutellum of embryos during and after the completion of the early stages of germination, and the sole CAT isozyme detectable in mature pollen, milky endosperm, and scutella of embryos during early kernel development (Chandlee and Scandalios, 1984; Wadsworth and Scandalios, 1989; Acevedo and Scandalios, 1990; Scandalios, 1990). Previous analyses of RNA and enzyme accumulation in excised immature maize

embryos showed that *Cat1* transcript accumulation and enzyme activity rapidly increased in the presence of exogenous ABA (Williamson and Scandalios, 1992a). In developing kernels of the ABA-deficient maize viviparous mutant *vp5*, lower accumulation of *Cat1* RNA correlated with lower measured endogenous ABA. In the maize *vp1* mutant, which is morphologically insensitive to ABA, accumulation of *Cat1* RNA paralleled normal changes in measured amounts of endogenous ABA. The response of *Cat1* to endogenous and exogenous ABA in mutant and wild-type *vp1* and *vp5* sibling embryos supported the hypothesis that, unlike *E<sub>m</sub>* and maize *glb* genes, the *Vp1* transcriptional activator does not affect the ABA-mediated expression of *Cat1* in developing embryos.

Although recent research has elucidated various elements of the ABA-mediated regulation of several genes (Hetherington and Quatrano, 1991), many important aspects of this regulation remain unresolved. The seed-specific transcriptional activator Vp-1 enhances the ABA-mediated expression of a number of embryogenesis-specific genes, and the involvement of the *SphI* consensus (CATGCAT) (McCarty et al., 1991) and the GT consensus ABA response element in the maize *C1* gene (Hattori et al., 1992) have been described. Using a rice transient expression system (Marcotte et al., 1989; Pla et al., 1993), the G-box consensus sequence *Em1a* was shown to control ABA-regulated expression of wheat *E<sub>m</sub>* and maize *rab28* promoter constructs. It has also been demonstrated that the Leu-zipper (B-zip) DNA binding protein EmBP-1 (Guiltinan et al., 1990) binds to this G-box consensus sequence. However, no qualitative differences in binding of nuclear proteins to ABA-regulated promoters in response to ABA have been demonstrated.

In this paper we present evidence for the differential binding of a 20-kD nuclear protein (CAT1BP-20) to a 28-bp region of the ABA-regulated *Cat1* promoter containing the G-box consensus sequence *Em1a*. This protein appears in nuclear extracts of immature maize embryos incubated in the absence of ABA but is absent, or present in reduced amounts, in immature embryos incubated in the presence of exogenous ABA. Large amounts of a 20-kD protein also appear in nuclear extracts of scutella of germinated mature maize em-

<sup>1</sup> This work was supported, in part, by grants from the United States Environmental Protection Agency (R819360) and National Science Foundation (DCB-9101238) to J.G.S.

<sup>2</sup> Present address: Department of Horticultural Science, Box 7609, North Carolina State University, Raleigh, NC 27695–7609.

\* Corresponding author; fax 1–919–515–3355.

Abbreviations: CAT, catalase protein; *Cat*, catalase gene; DPI, days postimbibition; DPP, days postpollination; *E<sub>m</sub>*, early methionine-labeled polypeptide; GM, growth medium; GUS,  $\beta$ -glucuronidase.

bryos at a time when exogenous application of ABA no longer up-regulates *Cat1* RNA expression. Taken together, these data suggest that CAT1BP-20 functions as an ABA-mediated repressor of *Cat1* expression.

## MATERIALS AND METHODS

### Plants and Plant Growth Conditions

The maize (*Zea mays* L.) inbred lines W64A and R6-67 maintained in this laboratory were used in these studies. Embryos were manually dissected from developing kernels of greenhouse-grown plants or from germinated mature kernels. Excised embryos were incubated on plates consisting of GM or GM supplemented with filter-sterilized ABA. Unsupplemented GM contained modified Murashige and Skoog salts (Murashige and Skoog, 1962; ammonium and potassium nitrate deleted, chelated iron added), 2% Suc, 0.9% T.C. Agar (Carolina Biological Supply Co., Burlington, NC), 0.1% casein hydrolysate (vitamin free, one-half salt). When assessing the effect of exogenous ABA, excised embryos were incubated overnight (14 h) in the dark at 25°C. After treatment, scutella (embryo minus axis) were manually isolated, frozen in liquid nitrogen, and stored at -70°C.

### Preparation of Plates

All ABA supplements were prepared by appropriate dilutions (using 0.1 M KOH) of a 10<sup>-2</sup> M stock of a racemic mixture of ABA (Sigma A-1049) dissolved in 0.1 M KOH. Because only the *R* enantiomer of ABA is active, effective concentrations of ABA are 0.5 times the indicated total concentrations. In embryo dose-response studies GM was supplemented with either KOH alone (-ABA) or the indicated dilution of the racemic ABA stock. Based on previous *Cat1* and *E<sub>m</sub>* dose-response studies (Williamson and Scandalios, 1992a), ABA-supplemented plates (+ABA) for all other analyses contained 10<sup>-4</sup> M racemic ABA (5 × 10<sup>-5</sup> M *R* ABA).

### RNA Analyses

Total RNA was isolated from tissue samples by a modification of a cold phenol extraction method (Williamson et al., 1985). For northern analysis of transcript accumulation, total RNA (20 µg) from each sample was separated on denaturing 1.2% agarose gels and transferred to nitrocellulose (Sambrook et al., 1989). The resulting blots were hybridized with a <sup>32</sup>P-labeled *Cat1* sequence-specific probe (Redinbaugh et al., 1988) as described (Sambrook et al., 1989). Following autoradiography of the resulting blot, probe was removed from the filters by repeated washes in boiling 0.1× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% (w/v) SDS. Filters were then reprobed with <sup>32</sup>P-labeled insert DNA from clone p1015 (Williamson et al., 1985) containing the entire coding sequence of the ABA-regulated *E<sub>m</sub>* polypeptide and subsequently with a DNA fragment from clone pHA2 containing an 18S rDNA sequence (Jorgensen et al., 1987).

### Nuclear Protein Extracts and Band-Shift Assays

Mature embryos were manually excised from seed of the maize inbred line W64A 5 DPI and from immature embryos

of seed 23 DPP. Excised immature embryos were also incubated on +ABA and -ABA plates as described above. Scutella were isolated from treated and untreated embryos and nuclear proteins were extracted as described (Guiltinan et al., 1990). Probes were prepared by end labeling either the 188-bp *HpaII* fragment from the *Cat1* promoter containing the G-box consensus sequence (see Fig. 3) or a synthetic 28-bp double-stranded oligonucleotide spanning this same sequence. Unlabeled competitor DNAs used included the G-box oligonucleotide described above, the human *Oct-1* (Goding and O'Hare, 1989), or the Epstein-Barr virus EBNA-1 (Ambinder et al., 1991) protein binding sequences (Pharmacia), and the nonspecific competitor poly(dI-dC). Binding reactions (20 µL) contained 5 to 10 fmol of end-labeled DNA (5,000–10,000 cpm), 5 µg of poly(dI-dC) (Boehringer-Mannheim), 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 10% glycerol, 0.05% Nonidet P-40, and 30- to 100-fold molar excess competitor DNA. Reactions were initiated by the addition of nuclear protein extract (5–10 µg) and incubated at room temperature for 20 to 30 min. Samples were then separated by electrophoresis on 5 to 7% single percentage or 4 to 12% precast gradient polyacrylamide gels (Bio-Rad) in Tris-acetate EDTA (Guiltinan et al., 1990), Tris-borate EDTA, or Tris-Gly buffer (Kawagoe and Murai, 1992). Gels were dried and bound and free probe was visualized by autoradiography.

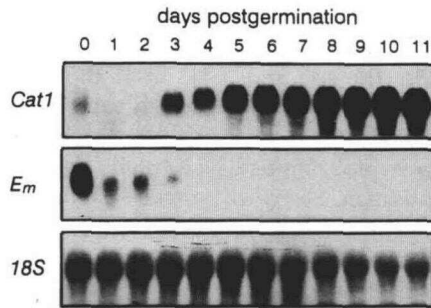
### Southwestern Blot Analysis

Scutella were isolated from treated and untreated embryos and nuclear proteins were extracted as described (Guiltinan et al., 1990). Nuclear proteins were separated by electrophoresis on 4 to 12% precast gradient SDS-polyacrylamide gels (Bio-Rad) in Tris-Gly-SDS buffer (Laemmli, 1970). Separated proteins were renatured and blotted to nitrocellulose as described (Wellstein et al., 1991). After transfer, blots were blocked in three washes containing 25 mM Tris (pH 7.5), 5% dry milk, 10% glycerol, 2.5% Nonidet P-40, 0.1 mM DTT, and 150 mM NaCl for 45 min at 25°C. Blots were then hybridized for 16 h at 25°C in binding buffer containing 5 × 10<sup>5</sup> cpm/mL end-labeled probe DNA prepared as described for band-shift assays. Hybridizations were conducted both with and without the same unlabeled competitor DNAs used for band-shift assays. Binding buffer contained 10 mM Tris (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM DTT, 8% glycerol, 0.125% dry milk, 5 mM MgCl<sub>2</sub>, and 10 µg/mL poly(dI-dC).

## RESULTS

### Accumulation of *Cat1* Transcript in Scutella of Germinated Maize Embryos

Extending our previous analyses of the regulation of maize *Cat1*, we examined the accumulation of *Cat1* RNA in the scutella of mature embryos at 0 through 11 DPI. Probing total RNA blots, a decrease in *Cat1* RNA accumulation was observed between 0 and 2 DPI followed by a dramatic increase between 3 and 11 DPI (Fig. 1). In contrast, accumulation of the ABA-regulated *E<sub>m</sub>* message decreased between 0 and 3 DPI and subsequently became undetectable. In separate experiments (A. Acevedo and J.G. Scandalios,



**Figure 1.** Accumulation of *Cat1* transcript in scutella of germinated maize embryos. Embryos were excised from germinated kernels of the maize inbred line W64A at 0 to 11 DPI (postgermination); total RNA was isolated, and accumulation of *Cat1* transcript in each sample was determined by northern blot analysis. *Cat1* probe was removed from the filters, and blots were reprobed with insert DNA containing the coding sequence of the ABA-regulated *Em* polypeptide and subsequently with a DNA fragment containing an 18S ribosomal sequence to ensure similar loading and transfer.

unpublished data), endogenous ABA content in scutella of germinated maize embryos was determined by tracer competition immunoassay as previously described (Williamson and Scandalios, 1992a). Measured ABA content was low at 0 DPI, decreased to undetectable amounts by 3 DPI, and remained below the level of detection through 11 DPI. Therefore, the large increase in *Cat1* RNA accumulation observed between 3 and 11 DPI was not due to increasing endogenous ABA. This implies that, in contrast to its response to ABA during embryogenesis, *Cat1* may be responding to a developmental signal other than ABA during seedling development.

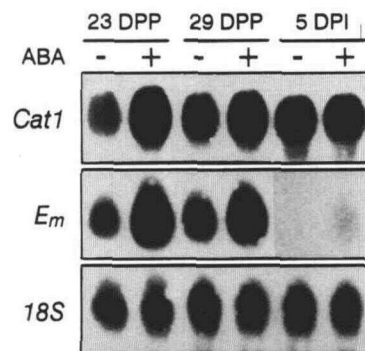
#### Effect of Exogenously Applied ABA on the Accumulation of *Cat1* RNA in the Scutella of Mature Germinated Maize Embryos

The previous observation led us to evaluate whether *Cat1* retains the ability to respond to ABA after the developmental shift from embryogenesis to germination. We first compared the response of immature (23 DPP), mature, unimbibed (29 DPP), and mature, germinated (5 DPI) W64A embryos to ABA. Excised embryos from each developmental time point were incubated for 14 h at 25°C in the dark on GM or GM supplemented with ABA. At the conclusion of the treatment, scutella were separated from embryo axes, and accumulation of the *Cat1* and *Em* transcripts in the scutella was determined by northern analysis of total RNA. Accumulation of both the ABA-regulated *Em* and *Cat1* transcripts increased in the presence of ABA in scutella of immature embryos (23 DPP) (Fig. 2). In contrast, in scutella of mature embryos (5 DPI), *Cat1* accumulation was unchanged. In addition, although total *Em* transcript accumulation was much lower than in immature embryos, amounts of *Em* RNA did increase in the presence of exogenous ABA. In a companion experiment, scutella of R6-67 embryos incubated on plates supplemented with doses of ABA ranging from 0 to  $10^{-2}$  M ABA showed no increase in *Cat1* RNA accumulation with increasing ABA (not shown).

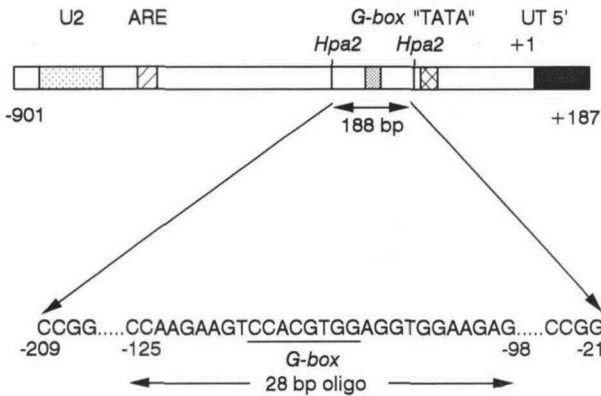
Data in the previous section suggest that *Cat1* may be responding to a developmental signal other than ABA after germination. These data suggest that, in addition, *Cat1* may also be less sensitive to regulation by exogenous ABA during this developmental stage.

#### Band-Shift Analysis of Proteins Binding to the *Cat1* Promoter in Nuclear Extracts of Scutella from Germinated and Developing Embryos

Sequence analysis of a maize *Cat1* genomic clone (Guan and Scandalios, 1993) showed that the *Cat1* promoter region contains an appropriately situated putative ABA response element (the G-box consensus sequence *Em1a*) but lacks both the Vp-1 response element (*SphI* consensus) (McCarty et al., 1991) or the GT consensus ABA response element found in the maize *C1* gene (Hattori et al., 1992). A 188-bp *HpaII* fragment in the *Cat1* promoter proximal region contains the *Em1a* sequence but no other identified consensus sequences (Reinbothe et al., 1992) (Fig. 3). In previous work a *Cat1*-GUS promoter fusion utilizing this 188-bp *HpaII* fragment directed GUS expression that paralleled measured endogenous ABA in developing seed of transgenic tobacco (Guan and Scandalios, 1993). The *Em1a* consensus has also been shown to control ABA-regulated expression of wheat *Em* and maize *rab28* promoter constructs in a rice transient expression system (Marcotte et al., 1989; Pla et al., 1993). Because *Cat1* appears to be regulated by ABA during embryogenesis but not after germination, it was of interest to see if there was differential binding of proteins to the promoter region containing the *Em1a* consensus. Therefore, assays were performed to determine if the observed shift in control of *Cat1* expression between embryogenesis and early postgermination is paralleled by a shift in proteins binding this region of the *Cat1* promoter. A 28-bp double-stranded oligonucleotide



**Figure 2.** Accumulation of *Cat1* transcripts in the scutella of immature and germinated mature maize embryos in the presence and absence of exogenous ABA. Embryos were excised from developing kernels of the maize inbred line W64A at 23 and 29 DPP and from mature germinated seed at 5 DPI. Excised embryos were incubated for 14 h in the dark on GM alone (–) or GM supplemented with  $10^{-4}$  M R/S racemic ABA (+). Total scutellar RNA was extracted from each treatment, and steady-state levels of the *Cat1* and *Em* transcripts were determined by northern blot analysis. Filters were subsequently reprobed with a DNA fragment containing an 18S ribosomal sequence to ensure similar loading and transfer.



**Figure 3.** Diagram of *Cat1* promoter sequences used in these studies. Probes used in these experiments were prepared by end labeling the indicated 188-bp *Hpa*I *Cat1* promoter fragment and a 28-bp synthetic oligonucleotide, both of which span the G-box consensus sequence *Em1a*. Sequences homologous to U2 snRNA (U2) and mammalian antioxidant response element (ARE) (Rushmore et al., 1991) are indicated.

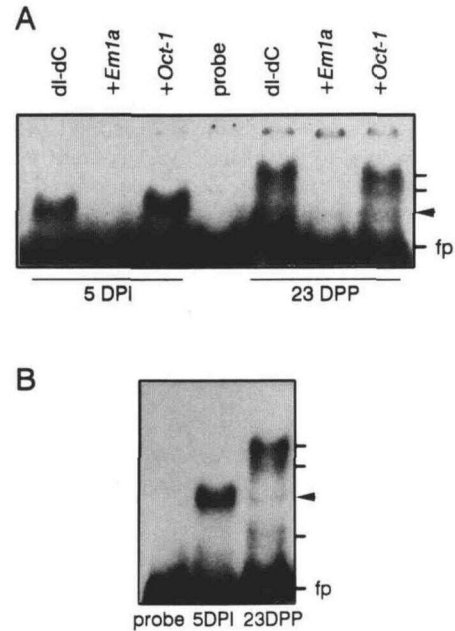
spanning the *Em1a* consensus sequence was also synthesized to localize binding more precisely to this region (Fig. 3).

For these analyses, scutella were isolated from untreated germinated embryos of the maize inbred line W64A at 5 DPI and from immature embryos at 23 DPP. Total nuclear proteins were extracted as described (Guiltingan et al., 1990), and band-shift assay conditions previously established for the *Em1a* consensus (Marcotte et al., 1989; Pla et al., 1993) were used to assay the ability of these nuclear protein extracts to bind the two *Cat1* promoter region probes. Both probes bound to what appear to be the same sets of proteins in corresponding extracts from germinated (5 DPI) and developing (23 DPP) embryos (Fig. 4). However, a change in the DNA binding complexes is apparent between these two developmental time points. For example, the major binding complex present at 5 DPI is barely detectable at 23 DPP. Instead, two lower-mobility bands represent the major complexes present at 23 DPP. Competition experiments (Fig. 4A) showed that although the unlabeled 28-bp *Em1a*-containing oligonucleotide competed with the 188-bp *Cat1* promoter fragment for binding proteins in both 5-DPI and 23-DPP samples, *Oct-1* and other random oligonucleotides did not. Using either the 28-bp *Em1a*-containing oligonucleotide or the 188-bp *Cat1* promoter fragment probe gave essentially the same band-shift pattern.

#### Southwestern Blot Analyses of Nuclear Proteins from Scutella of Immature and Germinated Embryos Binding to *Cat1* Promoter DNA

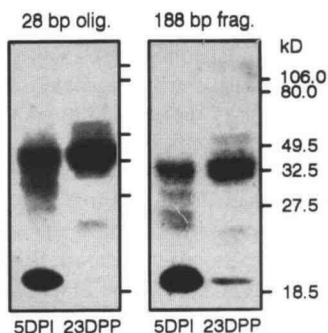
To further characterize this apparent shift in promoter binding proteins, complementary southwestern blot analyses (Wellstein et al., 1991) were performed. Nuclear proteins prepared above were first separated by SDS-PAGE, renatured, and blotted to nitrocellulose. Blotted nuclear proteins from 5-DPI and 23-DPP scutella were then probed with labeled *Cat1* promoter 188-bp fragment and the labeled 28-

bp oligonucleotide spanning the *Em1a* consensus. A protein(s) of 32- to 36-kD molecular mass bound both probes in all lanes (Fig. 5). The size and behavior of these proteins are consistent with those of the previously reported *Em* binding protein (EmBP-1) (Guiltingan et al., 1990), which, like other B-Zip proteins, probably binds as a dimer to the *Em1a* consensus. In addition, a smaller protein of approximately 20-kD molecular mass strongly bound both probes in samples from germinated tissues (5 DPI) but was absent, or present at greatly reduced amounts, in scutella of immature embryos (23 DPP). Several additional proteins ranging in size from 25 to 30 kD and present only in samples from germinated tissues (5 DPI) also bound both probes (Fig. 5). Thus, of the apparent differences in DNA binding proteins, none appear to be the *Em* binding protein (EmBP-1) previously described (Guiltingan et al., 1990).



**Figure 4.** Band-shift analysis of nuclear protein extracts from scutella of immature and germinated embryos. Embryos were excised from germinated kernels of the maize inbred line W64A 5 DPI and from developing kernels 23 DPP. Embryo axes were removed, and total nuclear proteins were isolated from the scutella. A, Binding reactions containing either end-labeled 188-bp *Hpa*I *Cat1* promoter fragment alone (probe; Fig. 3) or probe incubated in the presence of nuclear extracts (5  $\mu$ g) from either 5-DPI or 23-DPP scutella were separated on 7% polyacrylamide Tris-acetate EDTA gels. Reactions also contained 30-fold excess poly(dl-dC) alone (dl-dC) or in combination with unlabeled 28-bp *Em1a* oligonucleotide (+*Em1a*) or unlabeled *Oct-1* DNA binding consensus (+*Oct-1*) as competitor DNA. Migration of free probe (fp) and major retarded bands are indicated (—). The major band enhanced in the absence of ABA is indicated by an arrowhead. B, Binding reactions containing either end-labeled 28-bp *Em1a* oligonucleotide alone (probe; Fig. 3) or probe incubated in the presence of nuclear extracts (5  $\mu$ g) from either 5-DPI or 23-DPP scutella were separated on 7% polyacrylamide Tris-acetate EDTA gels.





**Figure 5.** Southwestern blot analysis of DNA binding proteins present in nuclear extracts of scutella of immature and germinated embryos. Embryos were excised from germinated kernels of the maize inbred line W64A 5 DPI and from developing kernels 23 DPP. Embryo axes were removed, and total nuclear proteins were isolated from scutella. Nuclear extracts were separated on 12% SDS-PAGE gels, renatured, and blotted to nitrocellulose. Blots were then probed with either end-labeled oligonucleotide or end-labeled promoter fragment (Fig. 3), and proteins binding labeled DNA were visualized by autoradiography. Migration of molecular mass standards is indicated.

#### Band-Shift Analyses of Nuclear Proteins from Scutella of ABA-Treated and Untreated Immature Embryos That Bind *Cat1* Promoter DNA

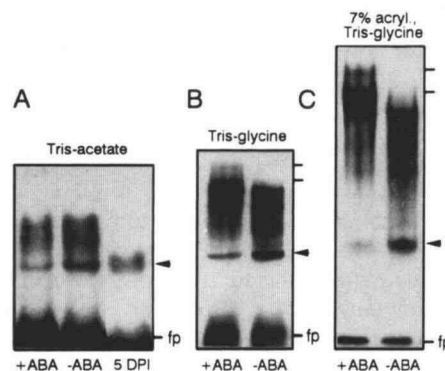
Because *Cat1* is regulated by ABA in the scutella of immature embryos, one might expect differential *Cat1* promoter binding using proteins isolated from nuclei of scutella of ABA-treated and ABA-untreated embryos. Therefore, band-shift and southwestern analyses were used to assess *Cat1* promoter binding proteins in the scutella of ABA-treated and -untreated immature embryos and compare them to binding proteins present in immature and germinated embryos.

Excised immature embryos from 21-DPP kernels were incubated for 14 h on unsupplemented GM (-ABA) and on GM supplemented with ABA (+ABA) (Williamson and Scandalios, 1992a). After treatment, total nuclear proteins were extracted from scutella, and band-shift assays were performed as described above (Guiltinan et al., 1990; Pla et al., 1993). Under these conditions no clear binding differences were detected in +ABA and -ABA extracts (Fig. 6A). To determine if different gel conditions allowed resolution of binding differences, samples were also separated on 5 and 7% acrylamide Tris-acetate EDTA gels, 4 to 12% gradient Tris-acetate EDTA gels, and 6% and 4 to 12% gradient Tris-borate EDTA gels. In addition, varying concentrations of nuclear protein and competitor DNAs [*Oct-1* and poly(dI-dC)] were used in the binding reactions themselves. However, no ABA-dependent differences in banding patterns were seen using any of these protocols (not shown), although several distinct banding differences were resolved using a Tris-Gly gel shift system (Kawagoe and Murai, 1992) (Fig. 6, B and C). Of the several types of gels used, 7% single percentage polyacrylamide gels gave the clearest separation (Fig. 6C). Comparing the complexes formed with -ABA extracts to those formed with +ABA extracts, we see a general shift toward higher mobility. Most notable was the increase in the

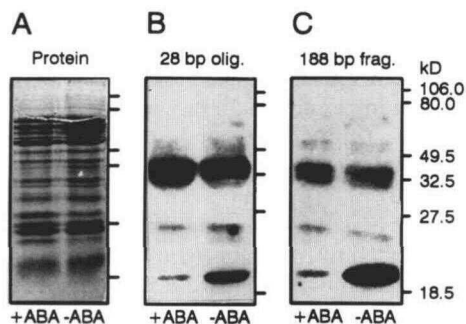
absence of ABA of the high-mobility complex corresponding to the postgermination-specific (5 DPI) complex previously seen (Figs. 4 and 7A). Maximum separation was achieved with no change in banding pattern when gels were not pre-electrophoresed. Titration series indicated that observed differences were not due to simple protein concentration differences (not shown). To demonstrate that higher protein concentrations were not generating the formation of higher-order (lower mobility) complexes in the +ABA lanes, the band-shift assays shown in Figure 6 were performed using 10  $\mu$ g of -ABA extract versus 5  $\mu$ g of +ABA extract.

#### Southwestern Analyses of Nuclear Proteins from ABA-Treated and ABA-Untreated Excised Embryos That Bind *Cat1* Promoter Sequences

In parallel experiments these same nuclear protein extracts were separated by SDS-PAGE, renatured, and blotted to nitrocellulose for southwestern analyses (Wellstein et al., 1991). Coomassie-stained protein gels showed no obvious change in banding pattern between +ABA and -ABA samples (Fig. 7A). Blots probed with labeled 28-bp oligonucleotide and labeled 188-bp *Cat1* promoter fragment both appeared to bind a subset of the nuclear proteins (Fig. 7) seen above (Fig. 5). Again, a protein(s) of 32- to 36-kD molecular mass bound both probes in all lanes (Fig. 7). In addition, a protein of approximately 20-kD molecular mass binding both probes was enhanced in samples from untreated (-ABA) tissues but was absent, or present in much lower amounts,



**Figure 6.** Band-shift analysis of nuclear protein extracts from scutella of +ABA- and -ABA-treated embryos. Embryos were excised from germinated kernels of the maize inbred line W64A 23 DPP and incubated for 14 h in the dark on GM alone (-) or GM supplemented with  $10^{-4}$  M *R/S* racemic ABA (+). Embryo axes were removed, and total nuclear proteins were isolated from the scutella. Probes used in these experiments were prepared by end labeling a 28-bp synthetic oligonucleotide spanning the G-box consensus sequence *Em1a* (Fig. 3). Binding reactions containing the end-labeled 28-bp *Em1a* oligonucleotide incubated in the presence of nuclear extracts from either +ABA- or -ABA-treated scutella from 21-DPP immature embryos (10 and 5  $\mu$ g, respectively) were separated on 7% polyacrylamide Tris-acetate EDTA gels (A), 4 to 12% gradient polyacrylamide Tris-Gly gels (B), or 7% polyacrylamide Tris-Gly gels (C). Migration of free probe (fp) and major low-mobility complexes are indicated (-). The major band enhanced in the absence of ABA is indicated by an arrowhead.



**Figure 7.** Southwestern blot analysis of DNA binding proteins present in nuclear extracts of scutella from +ABA and -ABA-treated embryos. Embryos were excised from germinated kernels of the maize inbred line W64A at 23 DPP and incubated for 14 h in the dark on GM alone (-) or GM supplemented with  $10^{-4}$  M *R/S* racemic ABA (+). Embryo axes were removed, and total nuclear proteins were isolated from the scutella. Nuclear extracts were separated on 12% SDS-PAGE gels, renatured, and blotted to nitrocellulose. A, Coomassie-stained proteins from +ABA and -ABA-treated samples. Blotted proteins were probed with either end-labeled oligonucleotide (B) or end-labeled promoter fragment (C) (Fig. 3). Blots were rinsed free of excess probe, and proteins binding labeled promoter DNA were visualized by autoradiography. Migration of molecular mass standards is indicated.

in scutella of ABA-treated embryos (+ABA). The postgermination-specific 25- to 30-kD bands previously seen were not present in nuclear protein extracts from scutella of either +ABA or -ABA-treated embryos. As above, the apparent difference in DNA binding proteins did not appear to be the approximately 34-kD  $E_m$  binding protein ( $EmBP-1$ ) previously described (Guiltinan et al., 1990).

## DISCUSSION

In a previous study we described the up-regulation of *Cat1* RNA accumulation in scutella of immature maize embryos in the presence of ABA (Williamson and Scandalios, 1992a). In scutella of mature germinated embryos, however, the patterns of accumulation of *Cat1* RNA and the ABA-regulated  $E_m$  message were quite different (Fig. 1). Although endogenous ABA and the ABA-regulated  $E_m$  message are very low after germination, *Cat1* RNA is quite abundant. During the first few DPI, RNA for both decreased in parallel with the continuing decrease in endogenous ABA begun in late embryogenesis. The  $E_m$  transcript decreased to undetectable amounts by 4 DPI, where it remained through 11 DPI (the latest time point examined). However, at approximately 3 DPI *Cat1* RNA began to increase dramatically and continued to do so through 11 DPI, even though ABA was undetectable after approximately 3 DPI. This led us to determine if *Cat1* could still be up-regulated by exogenous application of ABA in scutella of mature maize embryos. In these experiments accumulation of both *Cat1* and  $E_m$  RNA was markedly less sensitive to exogenous ABA than in immature embryos (Fig. 2) (Williamson and Scandalios, 1992a). The amounts of  $E_m$  induction in scutella of germinated seedlings were comparable to the ABA-mediated induction previously seen in

scutella of immature maize *vp1* mutant embryos (Williamson and Scandalios, 1992a). Because *Vp1* expression itself is seed specific, the low amount of ABA-mediated induction of  $E_m$  in germinated scutella may represent that small portion of  $E_m$  induction that is not *Vp1* mediated.

Given the high expression of *Cat1* in scutella during early seedling growth, its decreased sensitivity to ABA does not seem to be due to changes in chromatin structure producing decreased promoter accessibility. Instead, this implies that a "signal" other than ABA now mediates at least a major portion of *Cat1* expression. A similar shift in catalase gene response during the transition from embryogenesis to germination and growth was previously reported (Williamson and Scandalios, 1992b, 1993). In that study we saw differential response of catalases to toxin-containing extracts of *Cercospora* mycelia in germinated and developing maize scutella. Combined with the observations described above, we suggest that specific *trans*-acting factors involved in catalase gene expression are present only at particular developmental stages. Thus, depending on the availability of such factors, *Cat1* response to the same signal (in this case ABA) could be quite different during various developmental stages. This supports the inference that catalase promoters perhaps contain multiple control elements and that at least some of these control elements are tissue-, stage-, or signal-specific (i.e. control may be hierarchical). Such complex control of expression perhaps reflects the specific functions of these genes in cellular responses to oxidative stress.

It seemed likely, therefore, that this complex control of expression is reflected in changes in *Cat1* promoter binding proteins. It was shown (Guan and Scandalios, 1993) that the *Cat1* promoter has an appropriately positioned G-box consensus sequence (*Em1a*) (Fig. 3) and that expression of a minimal *Cat1* promoter-GUS fusion containing this sequence parallels changes in endogenous ABA in developing seed of transgenic tobacco. The G-box consensus *Em1a* has been shown to mediate ABA-regulated expression of wheat  $E_m$  and maize *rab28* promoter constructs in rice protoplasts (Marcotte et al., 1989; Pla et al., 1993). Therefore, we examined nuclear extracts from scutella of both developing and germinating embryos for changes in proteins binding a 188-bp *Cat1* promoter fragment and a 28-bp oligonucleotide, both containing the G-box/*Em1a* consensus and flanking *Cat1* promoter DNA. Scutella were used for these studies because they are simple organs that are terminally differentiated fairly early in embryogenesis (Kiesselbach, 1949). As a result, changes in gene expression are simpler to attribute to signal-specific responses. In contrast, whole embryos or shoots are complex associations of tissues, all or parts of which are still developing. Signal-specific responses in these tissues, therefore, may be masked by mixed responses in component tissues or by overlying developmental changes.

Using band-shift assays to assess *Cat1* promoter binding proteins present at these two developmental time points, we saw a change in mobility of the major DNA-protein complexes formed in vitro (Fig. 4). These differences are analogous to changes in band-shift patterns observed in assays of nuclear proteins from whole maize embryos versus salt-stressed leaves using a G-box-containing oligonucleotide from the maize *rab28* gene (Pla et al., 1993). The changes

reported here, however, occurred in the same fully differentiated tissue at different times, rather than in two different tissues (i.e. they are stage- rather than tissue-specific). Using southwestern analysis, we observed a matching shift in specific protein bands that bound labeled promoter DNA (Fig. 5). A major 32- to 36-kD nuclear protein band was present in samples from scutella of both immature and germinated embryos. In contrast, a 20-kD nuclear protein as well as several other proteins ranging from 25 to 30 kD were significantly enhanced in extracts from germinated tissues.

Although it has been demonstrated that at least one specific protein binds the *Em1a* consensus (EmBP-1; Guiltinan et al., 1990) and that the *Em1a* consensus mediates the ABA response of several genes (Marcotte et al., 1989; Guiltinan et al., 1990; Pla et al., 1993), specific ABA-mediated changes in proteins binding the promoter of an ABA-regulated gene have not been reported. Because the mid-embryogenesis and postgermination time points assayed were effectively +ABA and -ABA, respectively, and *Cat1* has been shown to be regulated by ABA in the scutella of developing maize embryos, we also assayed for differential binding of proteins from +ABA- and -ABA-treated scutella to *Cat1* promoter sequences. Using the band-shift assay conditions described above, we saw no binding differences between nuclear extracts from scutella of +ABA- and -ABA-treated embryos. However, using a Tris-Gly gel system described by Kawagoe and Murai (1992), several differences were observed (Fig. 6). These changes were again paralleled by changes in specific protein bands that bound labeled promoter-DNA in southwestern analyses (Fig. 7). Although a 32- to 36-kD nuclear protein band was present in samples from scutella of both +ABA- and -ABA-treated embryos, a 20-kD nuclear protein (CAT1BP-20) was more abundant in extracts from -ABA tissues. The 25- to 30-kD bands found in germinating samples did not appear in extracts of either treatment, implying that the 20-kD protein is specifically induced or derepressed in the absence of ABA, whereas the others are postgermination/stage specific. This does not appear to be GF-14 (de Vetten et al., 1992), because GF-14 does not bind to the DNA directly, nor does it change in response to ABA. To our knowledge, this is the first report of ABA-mediated differential binding of a nuclear protein to an ABA-regulated promoter.

Although these differences in DNA binding may indeed reflect different proteins in the DNA binding complex, they may also be due to the modification of existing proteins (e.g. differential phosphorylation, covalent modification, specific cleavage, etc.). Extracts used in these studies were prepared without NaF, implying that these specific differences are not a result of differential protein phosphorylation. In addition, PMSF was used both in extraction and assay buffers, suggesting that if cleavage of proteins is occurring, it is occurring in vivo as part of the cell's response to the absence of ABA. In fact, although there is a clear change in the SDS-PAGE pattern of nuclear proteins isolated from embryogenic versus germinated embryos, there was no gross change in nuclear proteins in extracts from scutella of embryos incubated with or without ABA (Fig. 7A). Therefore, although the germination-specific 25- to 30-kD proteins may be degradation prod-

ucts, the 20-kD protein (CAT1BP-20) appears to be a specific response to the absence of ABA.

Although these studies indicate that there is differential ABA-mediated binding of a nuclear protein localized to a 28-bp region surrounding the *Em1a* consensus, they do not demonstrate that the *Em1a* consensus is itself involved. The results of our experiments do show that an additional *Cat1* promoter binding protein appears in nuclear extracts from scutella of embryos incubated in the absence of ABA. In light of this fact one might consider the possibility that at least one of the "ABA-specific" controlling factors for *Cat1* is a repressor rather than an activator. It ought to be profitable, therefore, to screen expression libraries prepared from RNA from scutella of -ABA embryos with our labeled G-box oligonucleotide using hybridization conditions previously shown to bind this sequence in gel southwestern analyses. The *E<sub>m</sub>* promoter binding protein EmBP-1 was selected similarly by probing an expression library prepared from RNA of ABA-treated embryos with a labeled *Em1a* oligonucleotide probe (Guiltinan et al., 1990). We also plan to affinity purify the complete G-box binding complex using appropriate synthetic oligonucleotides bound to a column matrix (Eisenberg et al., 1990). The purified complexes (from +ABA and -ABA nuclear proteins) could then be resolved by SDS-PAGE and differences in composition directly determined.

#### ACKNOWLEDGMENTS

We thank Drs. Marilyn Ehrenschaft, Ralph Quatrano, and Steve Spiker for valuable discussions and for reading the final manuscript, and Sheri Kernodle and Stephanie Ruzsa for expert technical assistance.

Received April 29, 1994; accepted August 8, 1994.

Copyright Clearance Center: 0032-0889/94/106/1373/08.

#### LITERATURE CITED

- Acevedo A, Scandalios JG (1990) Expression of the catalase and superoxide dismutase genes in mature pollen in maize. *Theor Appl Genet* 80: 705-711
- Ambinder RF, Mullen MA, Chang YN, Hayward GS, Hayward SD (1991) Functional domains of Epstein-Barr virus nuclear antigen EBNA-1. *J Virol* 65: 1466-1478
- Chandlee JM, Scandalios JG (1984) Analysis of variants affecting the catalase developmental program in maize scutellum. *Theor Appl Genet* 69: 71-77
- de Vetten NC, Lu GH, Ferl RJ (1992) A maize protein associated with the G-box binding complex has homology to brain regulatory protein. *Plant Cell* 4: 1295-1307
- Eisenberg S, Francesconi SC, Civalier C, Walker SS (1990) Purification of DNA-binding proteins by site-specific DNA affinity chromatography. *Methods Enzymol* 182: 521-529
- Goding CR, O'Hare P (1989) Herpes simplex virus Vmw65-octamer binding protein interaction: a paradigm for combinatorial control of transcription. *Virology* 173: 363-367
- Guan L, Scandalios JG (1993) Characterization of the catalase antioxidant defense gene *Cat1* of maize, and its developmentally regulated expression in transgenic tobacco. *Plant J* 3: 527-536
- Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. *Science* 250: 267-271
- Hattori T, Vasil V, Rosenkrans L, Hannah LC, McCarty DR, Vasil IK (1992) The *Viviparous-1* gene and abscisic acid activate the C1 regulatory gene for anthocyanin biosynthesis during seed maturation in maize. *Genes and Dev* 6: 609-618

- Hetherington AM, Quatrano RS** (1991) Mechanism of action of abscisic acid at the cellular level. *New Phytol* **119**: 9–32
- Jorgensen RA, Cuellar RE, Thompson WF, Kavanagh TA** (1987) Structure and variation in ribosomal RNA genes of pea. Characterization of a cloned rDNA repeat and chromosomal rDNA variants. *Plant Mol Biol* **8**: 3–12
- Kawagoe Y, Murai N** (1992) Four distinct nuclear proteins recognize in vitro the proximal promoter of the bean storage protein  $\beta$ -phaseolin gene conferring spatial and temporal control. *Plant J* **2**: 927–936
- Kiesselbach TA** (1949) The structure and reproduction of corn. University of Nebraska Agricultural Experiment Station Research Bulletin 161
- Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685
- Marcotte WR, Russell SH, Quatrano RS** (1989) Abscisic acid response sequences from the Em gene of wheat. *Plant Cell* **1**: 523–532
- McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK** (1991) The *viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* **66**: 895–905
- Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**: 473–497
- Pla M, Vilardell J, Guiltinan MJ, Marcotte WR, Niogret MF, Quatrano RS, Pagès M** (1993) The *cis*-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene *rab28*. *Plant Mol Biol* **21**: 259–266
- Redinbaugh MG, Wadsworth GJ, Scandalios JG** (1988) Characterization of catalase transcripts and their differential expression in maize. *Biochim Biophys Acta* **951**: 104–116
- Reinbothe C, Tewes A, Reinbothe S** (1992) Comparative molecular analysis of gene expression during plant embryogenesis: do evolutionarily conserved mechanisms control early plant development? *AgBiotech News and Information* **4**: 381N–397N
- Rushmore TH, Morton MR, Pickett CB** (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem* **266**: 11632–11639
- Sambrook J, Fritsch EF, Maniatis T** (1989) *Molecular Cloning: A Laboratory Manual*, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Scandalios JG** (1965) Subunit dissociation and recombination of catalase isozymes. *Proc Natl Acad Sci USA* **53**: 1035–1040
- Scandalios JG** (1990) Response of plant antioxidant defense genes to environmental stress. *Adv Genet* **28**: 1–41
- Scandalios JG, Tong W-F, Roupakias DG** (1980) *Cat3*, a third gene locus coding for a tissue-specific catalase in maize: genetics, intracellular location, and some biochemical properties. *Mol Gen Genet* **179**: 33–41
- Wadsworth GJ, Scandalios JG** (1989) Differential expression of the maize catalase genes during kernel development: the role of steady-state mRNA levels. *Dev Genet* **10**: 304–310
- Wellstein A, Dobrenski AF, Radonovich MN, Brady JF, Riegel AT** (1991) Purification of PO-B, a protein that has increased affinity for the pro-opiomelanocortin gene promoter after dephosphorylation. *J Biol Chem* **266**: 12234–12241
- Williamson JD, Quatrano RS, Cuming AC** (1985)  $E_m$  polypeptide and its messenger RNA levels are modulated by ABA during embryogenesis in wheat. *Eur J Biochem* **152**: 501–507
- Williamson JD, Scandalios JG** (1992a) Differential response of maize catalases to abscisic acid: *Vp1* transcriptional activator is not required for abscisic acid-regulated *Cat1* expression. *Proc Natl Acad Sci USA* **89**: 8842–8846
- Williamson JD, Scandalios JG** (1992b) Differential response of maize catalases and superoxide dismutases to the photoactivated fungal toxin cercosporin. *Plant J* **2**: 351–358
- Williamson JD, Scandalios JG** (1993) Response of the maize catalases and superoxide dismutases to cercosporin-containing fungal extracts: the pattern of catalase response in scutella is stage specific. *Physiol Plant* **88**: 159–166